Investigating Known and Novel Biomarkers for Risk Stratification in Cardiovascular Disease

by

Melody Chemaly
(PharmD, MSc)

A thesis presented to Ulster University for the degree of Doctor of Philosophy

School of Biomedical Sciences

Faculty of Life and Health Sciences (Stratified Medicine)

Ulster University at Coleraine

October 2017

Supervisors:

Dr Victoria McGilligan, Professor Aaron Peace, Professor Tony Bjourson

I confirm that the word count of this thesis is less tham 100,000 words

Contents

Acknowledgements ix
Declaration x
List of Papers xi
Important Definitions xii
Abstract 1
Abbreviations 2
Chapter 1 4
Introduction to Cardiovascular Disease Risk Assessment and Exploration of Novel Biomarkers 4
1.1 Introduction to Cardiovascular Disease 5
1.1.1 Atherosclerosis 6
1.1.2 Likelihood of recurrences of cardiac events after first event 8
1.2 Acute coronary syndrome (ACS) and myocardial infarction *(ESC guidelines 2015)* 10
1.2.1 Definition and diagnostic algorithm 10
1.2.2 STEMI *versus* NSTEMI 13
1.2.3 Unstable angina 13
1.2.4 Cardiac troponins 14
1.3 Risk of cardiovascular disease 15
1.3.1 Defining risk 15
1.3.2 History of risk 16
1.3.3 Traditional risk factors 16
1.3.4 Risk prevention 19
1.3.5 Risk scores 19
1.4 Biomarkers 33
1.4.1 Biomarker definition 33
1.4.2 Ideal biomarker 34
1.4.3 Single *versus* multiple biomarkers 37
1.4.4 Defining abnormal biomarker values 38
1.4.5 Laboratory factors in biomarker evaluation 39
1.4.6 Biomarker discovery 41
1.5 The need for new risk prediction tools: Exploring the TACE/TNFα pathway and new biomarkers using a proteomic approach 44
1.6 Hypothesis 46
1.7 Aims 46

1.7.1 Evaluate the value of proteins from TNFα inflammatory pathway in cardiovascular disease (CVD) risk assessment and stratification 46
1.7.2 Investigate the value of a panel combining 10 inflammatory markers in CVD risk assessment 47
1.7.3 Determine whether a proteomic approach combining 184 proteins could improve the current CVD risk assessment scores 47
1.7.4 Explore whether potential proteins are useful in further stratifying very high risk CVD participants in terms of recurrent major adverse cardiovascular events (MACE) 47
1.7.5 Assess whether candidate proteins for biomarker development are subject to experimental and technical variability 48
Chapter 2 49
Patients, Materials and Methods 49
2.1 Methods for participant recruitment 50
2.1.1 Participants recruited from Altnagelvin Area Hospital 50
2.1.2 Participants recruited among the Western Trust and Ulster University staff 52
2.1.3 Methods for scoring participants according to the SCORE risk chart (ESC guidelines) 52
2.1.4 Methods for recruiting follow up participants 55
2.2 Methods for blood sample collection 55
2.3 Methods for spinning, aliquoting and storing blood samples 58
2.4 Method for extracting proteins stored in M-PER™ Mammalian Protein Extraction Reagent (#78501) – ThermoFisher Scientific, (Illinois, USA) - in -80◦C 60
2.5 Method for quantifying proteins using the bicinchoninic acid (BCA) assay protein assay kit (23225) – Pierce Thermo Scientific, (Illinois, USA) 61
2.6 TACE ELISA 62
2.6.1 Method for measuring tumour factor necrosis alpha converting enzyme (TACE) protein levels in the plasma by ELISA - Human TACE ELISA Kit - RAB0003 SIGMA, (Missouri, USA) 62
2.6.2 Method for measuring tumour factor necrosis alpha converting enzyme (TACE) protein levels in preparations of extracted proteins by ELISA - Human TACE ELISA Kit - RAB0003 SIGMA, (Missouri, USA) 67
2.7 Soluble TNFR1 ELISA 68
2.7.1 Method for measuring soluble tumour necrosis factor receptor 1 sTNFR1/TNFRSF1A protein levels in the plasma by ELISA – Human TNF RI/TNFRSF1A Immunoassay R&D - DRT100 (Minneapolis, USA) 68
2.7.2 Method for measuring soluble tumour necrosis factor receptor 1 sTNFR1/TNFRSF1A protein levels in preparations of extracted proteins by ELISA - Human TNF RI/TNFRSF1A Immunoassay R&D - DRT100 (Minneapolis, USA) 71
2.8 Soluble TNFR2 ELISA 72
2.8.1 Method for measuring soluble tumour necrosis factor receptor 2 sTNFR2/TNFRSF1B in the plasma by ELISA – Human TNF RII/TNFRSF1B Immunoassay R&D - DRT200 (Minneapolis, USA) 72

2.8.2 Method for measuring soluble tumour necrosis factor receptor 2 sTNFR2/TNFRSF1B protein levels in preparations of extracted proteins by ELISA - Human TNF RII/TNFRSF1B Immunoassay R&D - DRT200 (Minneapolis, USA) 75
2.9 TIMP3 ELISA 76
2.9.1 Method for measuring TIMP3 protein levels in the plasma by ELISA- TIMP3 (MIG-5) Human ELISA -abcam ab119608 (Cambridge, UK) 76
2.9.2 Method for measuring TIMP3 protein levels in preparations of extracted proteins by ELISA - TIMP3 (MIG-5) Human ELISA -abcam ab119608 (Cambridge, UK) 79
2.10 Method for Meso Scale ELISA - Proinflammatory Panel 1 (human) Kit – IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α - MSD® MULTI-SPOT Assay System - K15049D (Maryland, USA) 80
2.11 Method for RNA extraction from samples stored in RNAlater® Stabilization solution using the RiboPure™ RNA Purification Kit - AM1924- Ambion (California, USA) 86
2.12 Method for making up cDNA using the Transcriptor First Strand cDNA Synthesis Kit – 04379012001 – Roche (Mannheim, Germany) 89
2.13 Method for real-time polymerase chain reaction using Roche Light cycler®480 II and the LightCycler® 480 probe Master reagents (04707494001) and probes – Roche (Mannheim, Germany) 90
2.14 Method for analysing samples using the Proximity Extension Assay (PEA) technology - Proseek® Multiplex provided by O-Link Proteomics (Uppsala, Sweden) 95
2.15 Methods for performing the colony forming assay (CFA) 102
2.15.1 Preparation of collagen coated 96-well plates 102
2.15.2 Preparation of endothelial colony forming cells (ECFCs) culture medium 102
2.15.3 White blood cell isolation 103
Chapter 3 105
TACE/ADAM17 and TNFα identify and further stratify individuals at very high risk of cardiovascular events 105
Abstract 106
3.1 Introduction 108
3.2 Participants and Methods 110
3.2.1 Demographic information 110
3.2.2 Measurement of TNFα plasma protein levels 111
3.2.4 Measurement of *TACE* gene expression 111
3.2.5 Measurement of plasma TACE plasma protein levels 111
3.2.6 Measurement of cell membrane bound TACE protein levels 111
3.2.7 Statistical methods 112
3.3. Results 113
3.3.1 Population Demographics 113
3.3.2 TNFα plasma levels were higher in VHR participants 116
3.3.3 *TACE* mRNA levels were higher in VHR participants 121

3.3.4 Cell membrane bound TACE protein levels were higher in VHR participants 122
3.3.5 TACE plasma levels were not significantly different between VHR and non-VHR participants 123
3.3.6 CRP plasma levels did not further differentiate between VHR participant sub-groups 125
3.3.7 *TACE* gene expression correlated positively with TNFα plasma levels and both proteins can predict cardiovascular risk 125
3.3.8 TACE and TNFα plasma levels did not correlate with angiographic outcome 128
3.4 Discussion 133
3.4.1 TNFα plasma and *TACE* mRNA levels identified and further stratified VHR participant sub-groups 133
3.4.2 Exploration of TACE plasma and cell membrane bound levels in individuals at various levels of cardiovascular risk 136
3.4.3 Strengths and Limitations 138
3.5 Conclusion 141
Chapter 4 142
TNFR1, TNFR2 and TIMP3 identify and further stratify individuals at very high risk of cardiovascular events 142
Abstract 143
4.1 Introduction 145
4.2 Participants and Methods 146
4.2.1 Demographic information 146
4.2.3 Measurement of TNFR1, TNFR2 and TIMP3 plasma protein levels 147
4.2.4 Measurement of *TIMP3* gene expression 147
4.2.5 Cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels 147
4.2.6 Statistical methods 147
4.3. Results 148
4.3.1 Population Demographics 148
4.3.2 TNFR1 plasma protein levels were higher in VHR participants 149
4.3.3 TNFR2 plasma protein levels were higher in VHR participants 153
4.3.4 TIMP3 plasma protein levels were not statistically different between VHR and non-VHR participants whereas *TIMP3* mRNA levels were lower in VHR participants 155
4.3.5 Cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels in VHR participants 156
4.3.6 TNFR1 and TNFR2 plasma protein levels correlated positively 158
4.3.7 CRP plasma levels did not further differentiate between VHR participant sub-groups 160
4.3.8 TNFR1, TNFR2 and TIMP3 plasma protein levels did not predict angiographic outcome 160

4.4 Discussion 164
4.5 Conclusion 170
Chapter 5 171
Evaluating the Value of a Proinflammatory Panel in Cardiovascular Risk Assessment 171
Abstract 172
5.1 Introduction 174
5.2 Participants and Methods 176
5.2.1 Demographic information 176
5.2.2 Measurement of inflammatory plasma protein levels 176
5.2.3 Measurement of *IL1B* gene expression 176
5.2.4 Statistical methods 176
5.3 Results 176
5.3.1 Populaition Demographics 176
5.3.2 INF-γ, IL-6 and IL-8 plasma protein levels were higher in VHR participants 178
5.3.3 INF-γ, IL-6, IL-8, IL12-p70 and IL-10 plasma protein levels differentiated between VHR participant subgroups 180
5.3.4 Assessing the value of a multimarker model in predicting cardiovascular risk. 185
5.3.5 A cluster analysis to further stratify VHR cohort using INF-γ, IL-6, IL-8 and TNFα plasma protein levels 185
5.3.6 CRP plasma levels did not further differentiate between VHR participant sub-groups 186
5.3.7 Correlations between biomarker protein levels 186
5.4 Discussion 193
5.5 Conclusion 199
Chapter 6 200
Biomarker Discovery set: 200
A proteomic approach for identifying individuals at various levels of cardiovascular risk 200
Abstract 201
6.1 Introduction 203
6.2 Participants and Methods 205
6.2.1 Demographic information 205
6.2.2 Method for analysing plasma samples using the Proximity Extension Assay (PEA) technology - Proseek® Multiplex provided by O-Link Proteomics (CVDII and CVDIII) 205
6.2.3 Statistical methods 205
6.3 Results and Discussion 206
6.3.1 Biomarkers of cardiovascular risk 207
6.3.2 Biomarkers differentiating between very high risk (VHR) participant subgroups 227
6.3.3 Multimarker approach in biomarker research and development 240

6.3.4 Strengths and limitations 241
6.4 Conclusion 241
Chapter 7 243
Biomarkers predicting MACE risk within one year of admission in individuals at very high cardiovascular risk 243
Abstract 244
7.1 Introduction 246
7.2 Participants and Methods 247
7.2.1 Demographic information 247
7.2.2 MACE follow up 247
7.2.4 Measurement of TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels 248
7.2.5 Measurement of *TACE* gene expression 248
7.2.6 Measurement of TNFα, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IL-13 plasma protein levels 248
7.2.7 Analysis of O-link Cardiovascular panel II and III (O-link Proteomics®) in plasma samples 248
7.2.8 Statistical methods 248
7.3 Results 249
7.4 Discussion 258
7.5 Conclusion 264
Chapter 8 265
Exploring Analytical and Technical Parameters during Biomarker Development 265
Abstract 266
8.1 Introduction 268
8.2 Participants and Methods 269
8.2.1 Demographic information 270
8.2.2 Participants blood sample collection 270
8.2.3 Measurement of soluble TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels 271
8.2.4 Measurement of TNFα, IFN-γ, IL-2, IL-6, IL-8, IL-10 and IL-12p70 plasma protein levels 272
8.2.5 Method for analysing samples using the Proximity Extension Assay (PEA) technology - Proseek® Multiplex provided by O-Link Proteomics (CVDII and CVDIII) 272
8.2.6 Statistical methods 272
8.3 Results and Discussion 273
8.5 Conclusion 286
Chapter 9 287
General Discussion and Future Work 287
9.1 Major Findings 288

9.2 General Discussion 290
9.2.1 The need for better prediction tools 290
9.2.2 Identifying patients at very high risk of cardiovascular events 290
9.2.3 Identifying CVD individuals at risk of MACE 298
9.2.4 Obstacles in translating biomarker research into clinical practice 309
9.2.5 Conclusions and Future Work: The promise of personalised medicine is possible with multimarker models 312
Appendices 315
I. Additional Findings 316
I.I Seasonal changes and biomarker levels 316
I.II Endothelial dysfunction and cardiovascular disease 317
III. Tables 320
IV. Literature Review 350
References 351

Acknowledgements

This thesis becomes a reality due to the help and support of many brilliant individuals who I would like to offer my deepest and most sincere gratitude to.

I would like to express my deepest thanks and sincere appreciation to my supervisor Dr Victoria McGilligan. Thank you for your inspiration, your help and support (at whatever time of the day or night), your unlimited patience, your constant encouragement and your endless kindness. Thank you for this great PhD opportunity that I would have never dreamed of being able to do. I couldn't have done it without you!

I would like to express my extreme gratitude towards Prof Aaron Peace whose passion for research is endless. I have learned so much from you and I would like to profoundly thank you for your constant encouragement and support during my PhD. Thanks for being an extraordinary guide throughout this project.

I would like to express my special gratitude towards Prof Tony Bjourson. Your ideas, inspirations, enthusiasm and dynamism have without a doubt enabled me to complete this thesis. Thank you for being an incredible mentor throughout this PhD.

I would also like to express my deepest gratitude towards Prof Denis Alexander. Thank you for your exceptional ideas and inspirations as well as your constant kind support which I appreciated immensely throughout my PhD.

I owe a deep sense of gratitude towards Dr Steven Watterson for his great help and support in the completion of this project. Thank you for your kindness and for your patience.

I would like to thank my mom and dad for your permanent love and support. May God bless you and keep you safe. Thank you for always being by my side and for supporting me. I won't be the person I am today without you. Thank you for always inspiring me to a better person, to be kind, understanding and forgiving. I love you very much!

Merci à mes frères Elie et Zahi. Elie, ton esprit créatif et ta curiosité scientiqfique est illimitée! Si seulement j'avais réusssi à te convaincre de faire une thèse aussi! Tu serais devenu le meilleur professeur d'aéronautique au monde. Mais je suis sûre que tu vas le devenir par un moyen ou un autre. Zahi, merci pour tes conseils et ton IT support! Tu m'as sauvé la vie pleins de fois quand mes progmames statistiques ne fonctionnaient pas. Tu es la personne la plus intelligente que j'ai jamais rencontré. Depuis qu'on étaient tous petits, tu ne cessait pas de nous dépasser, et non seulement en grandeur de taille. Je suis très fiere de mes deux frères! Je suis la soeur la plus chanseuse au monde et je vous aime beaucoup. Merci Stéphanie pour ta confiance en moi et ton amitié illimitée! Je t'aime beaucoup ma Saarti!

Jag vill tacka min underbara Juha. Tack för din kärlek och ditt stöd. Jag är så lycklig att få dig. Du har en stor vetenskaplig nyfikenhet och jag är säker på att du kommer att sluta göra en doktorand också. Du är min djupaste inspirationskälla. Jag älskar dig så mycket min älskling!

Merci Laura pour ton inspiration, ta gentillesse, ton amitié et ton support! Mon experience à Derry n'aurait été pas du tout la même sans toi! Je suis très contente d'avoir trouvé une amie qui partage les mêmes passions que moi et qui n'a pas de problème à regarder les films Disney plus que 100 fois! Merci pour tout!

Declaration

"I hereby declare that for a period of two years following the date on which the thesis is deposited in the library of Ulster University, the thesis shall remain confidential with access or copying prohibited. Following expiry of this period I permit the Librarian of Ulster University to allow the thesis to be copied in whole or in part without reference to me on the understanding that such authority applies to the provision of single copies made for study purposes or for the inclusion within the stock of another library. *This restriction does not apply to the British Library Thesis service (which, subject to expiry of the period of confidentiality, is permitted to copy the thesis on demand for loan or sale under the terms of separate agreement) nor to the copying or publication of the title and abstract of the thesis.* IT IS A CONDITION OF USE OF THIS THESIS THAT ANYONE WHO CONSULTS IT MUST RECOGNISE THAT THE COPYRIGHT RESTS WITH THE AUTHOR AND THAT NO QUOTATION FROM THE THESIS AND NO INFORMATION DEROVED FROM IT MAY BE PUBLISHED UNLESS THE SOURCE IS PROPERLY ACKNOWLEDGED".

List of Papers

I- Published in Archives of Cardiovascular Diseases

Chemaly M, McGilligan V, Gibson M, Clauss M, Watterson S, Alexander HD, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis. 2017 Dec;110(12):700–11.

II- Published in the European Heart Journal (Abstract)

Chemaly M, Gibson M, Watterson S, Bjourson T, McGilligan V, Peace A. P6229TACE gene expression and soluble receptors TNFRI and TNFRII levels identifies very high risk cardiovascular patients. Eur Heart J [Internet]. 2017 Aug 1 [cited 2018 Feb 6];38(suppl_1). Available from: https://academic.oup.com/eurheartj/article/38/suppl_1/ehx493.P6229/4087246

III- Published in Heart (Abstract)

Gallagher L, McCusker M, **Chemaly M**, Peace A, Gibson M, Bjourson AJ, et al. 54 The nlrp3 inflammasome and other related receptors as potential biomarkers in coronary artery disease (cad) and major adverse cardiac events (mace). Heart. 2017 Oct 6;103(Suppl 6):A31–2.

IV- Published in Current Atherosclerosis Reports

Abifadel, M., Elbitar, S., El Khoury, P., Ghaleb, Y., **Chémaly, M**., Moussalli, M.-L., Rabès, J.-P., Varret, M., and Boileau, C. (2014). Living the PCSK9 adventure: from the identification of a new gene in familial hypercholesterolemia towards a potential new class of anticholesterol drugs. Curr. Atheroscler. Rep. *16*, 439.

V- Published in Osteoarthritis Cartilage

McAllister, M. J., **Chemaly, M.,** Eakin, A. J., Gibson, D. S. & McGilligan, V. E. NLRP3 as a potentially novel biomarker for the management of osteoarthritis. *Osteoarthr. Cartil.* (2018). doi:10.1016/j.joca.2018.02.901

Important Definitions

Cardiovascular diseases (CVD) are a group of diseases affecting the blood vessels and include coronary artery disease (CAD), ischaemic stroke and peripheral arterial disease (PAD).

Coronary artery disease (CAD) is a progressive inflammation of the arterial wall caused by a passive build-up of cholesterol that could lead into myocardial infarction.

Myocardial infarction (MI) is caused by a reduction in the blood flow in one part of the heart causing muscle damage and could be due to the formation of a blood clot.

Acute coronary syndrome (ACS) are medical emergencies that group different types of MI. They include ST-segment elevation myocardial infarction (STEMI) which is characterised by a high increase in Troponins, non ST-segment elevation myocardial infarction (NSTEMI) which is characterised by a moderate increase in troponins and unstable angina (UA) with no increase in troponins.

Cardiovascular risk is assessed by established risk factors such as cholesterol levels, blood pressure levels, smoking status and the presence of diabetes and family history. In Europe, cardiovascular risk is assessed based on the SCORE risk chart.

Cardiovascular events are referred to within the literature as a group of events including ACS, strokes, the development of heart failure, cardiovascular deaths and the need for a coronary revascularisation.

Major adverse cardiovascular events (MACE) have been referred to within this work as recurrent cardiovascular events in an individual with an established CVD. These were defined as cardiovascular deaths, strokes, recurrent ACS, recurrent angina, stent restenosis and the recurrent need for addition revascularisation procedures

Revascularisation procedure aims to restore the blood flow to the heart by treating a blocked blood vessel. This can be achieved by stent implantation (known as percutaneous coronary intervention) or by placing new blood vessels around the existing blockage (also known as coronary artery bypass grafting).

A **biomarker** is defined as a biological marker that reflects a certain disease state and guides clinical decision.

Primary prevention is defined as a set of procedures that are put in place to control CVD risk factors to delay or prevent the onset of CVD.

Secondary prevention is defined is defined as a set of procedures that are put in place in an individual with an established CVD to prevent recurrent MACE.

Abstract

Biomarker research in cardiovascular disease (CVD) is increasingly challenging with very few biomarkers being adopted into clinical practice. CVD risk assessment scores are in place to guide primary and secondary prevention however, they require considerable improvement. At present, there is no blood test that can predict who is at higher risk of first or recurrent cardiovascular events. Since CVD is a complex inflammatory disease with several underlying biological pathways, one biomarker is unlikely to define an individual's cardiovascular risk. However, it is possible to improve the current risk assessment scores using a multimarker approach. To date no one has investigated the many known and potentially novel markers in individuals at various levels of CVD risk. Therefore, in this present work, 344 participants at various levels of CVD risk (according to the European Society of Cardiology risk score), were recruited to explore potential biomarkers for risk stratification.

Proteins belonging to the tumour necrosis factor alpha (TNFα) inflammatory pathway, which has previously been shown to play a major role in CVD initiation and complications, as well as novel proteins associated with atherosclerosis, plaque rupture and thrombosis were explored using ELISA, multiplex proximity extension assays (PEA) developed by Olink Proteomics®, MSD® MULTI-SPOT Assay System and quantitative real-time PCR. Results reveal a complex panel of markers that were able to identify individuals at very high risk of CVD. Furthermore, more specific panels of markers were discovered that were able to further stratify patients at very high risk of CVD according to their cardiac history and co-morbidities. The present investigation overall demonstrates that a combination of proteins from several inflammatory pathways are necessary to evaluate an individual's risk of first or recurrent cardiovascular events as well as the risk of developing CVD-associated co-morbidities and treatment response.

The blood biomarker panels discovered in this present work can be used to establish a unique proteomic disease signature for each individual recruited to the study. This will aid future long term prospective longitudinal studies in evaluating the clinical utility of such panels alone or in combination with current risk prediction tools.

Abbreviations

CVD: Cardiovascular Disease
CAD: Coronary artery Disease
MACE: Major Adverse Cardiovascular Events
MI: Myocardial Infarction
PCI: Percutaneous Coronary Intervention
CABG: Coronary Artery Bypass Grafting
BMI: Body Mass Index
GFR: Glomerular Filtration Rate
CRP: C Reactive Protein
ESC: European Society of Cardiology
EUROSCORE: European Systematic COronary Risk Evaluation (SCORE) Algorithm
PEA: Proximity Extension Assays
ELISA: Enzyme-Linked Immunosorbent Assay
Real-time PCR: Real Time Polymerase Chain Reaction
TNFα: Tumour Necrosis Factor Alpha
TACE: Tumour Necrosis Factor Alpha Converting Eenzyme
TNFR1: Tumour Necrosis Factor rReceptor 1
TNFR2: Tumour Necrosis Factor Receptor 2
TIMP3: Metalloproteinase Inhibitor 3
INF-γ: Interferon Gamma
IL-1β: Interleukin 1 Beta
IL-2: Interleukin 2
IL-4: Interleukin4
IL-6: Interleukin 6
IL-8: Interleukin 8
IL-10: Interleukin 10
IL-12p70: Interleukin 12 Hetoremer 70
MMP7: Matrix Metalloproteinase 7
LOX-1: Lectin-Type Oxidized LDL Receptor 1
MMP12: Matrix Metalloproteinase 12
GDF-15: Growth/Differentiation Factor 15
REN: Renin
CNTN1: Contactin 1
TRAP: Thrombospondin-Related Adhesive Protein
hOSCAR: Osteoclast-aAssociated Immunoglobulin-Like Receptor
CEACAM8: Carcinoembryonic Antigenrelated Cell Adhesion Molecule 8
PAPPA: Pappalysin-1
TNFRSF11A: Tumour Necrosis Factor Receptor Superfamily Member 11A
PON3: Paraoxonase
MARCO: Macrophage Receptor with Collagenous Structure
TNFSF13B: Tumour Necrosis Factor Ligand Superfamily Member
VSIG2: V-set and Immunoglobulin Domain Containing Protein 2
KIM1: Kidney Injury Molecule 1
TRAIL-R2: TNF Related Apoptosis-Inducing Ligand Receptor 2
RARRES2: Retinoic Acid Receptor Responder Protein 2

CCL16: C-C Motif Chemokine 16
IL-1RT1: Interleukin-1 Receptor Type 1
TFF3: Trefoil Factor 3
ADM: Adrenomedullin
CXCL16: C-X-C Chemokine Motif 16
TR: Transferrin Receptor Protein 1
ADAMTS13: A Disintegrin And Metalloproteinase With Thrombospondin Motifs 13
TRAIL-R2: TNF-Related Apoptosis-Inducing Ligand Receptor 2
MMP3: Matrix Metalloproteinase 3
LTBR: Lymphotoxin-Beta Receptor
TNFRSF14: Tumour Necrosis Factor Receptor Superfamily Member 14
PDL2: Programmed Cell Death 1 Ligand 2
GDF-15: Growth/Differentiation Factor 15
EPHB4: Ephrin Type-B Receptor 4
IL2RA: Interleukin-2 Receptor Subunit Alpha
SHPS1: Tyrosine-Protein Phosphatase Non-Receptor Type Substrate 1
IDUA: Alpha-L-Iduronidase
AGRP: Agouti Related Protein
IL-1RT2: Interleukin 1 Receptor 2
CCL22: C-C Motif Chemokine 22
KLK6: Kallikrein-6
THBS2: Thrombospondin 2
IL-4RA: Interleukin-4 Receptor Subunit Alpha
PI3: Elafin
PLC: Perlecan
REN: Renin
IL-6: Interleukin 6
VSIG2: V-Set And Immunoglobulin Domain-Containing Protein 2
IGFBP2: Insulin-Like Growth Factor-Binding Protein 2
uPAR: Urokinase Plasminogen Activator Surface Receptor
FGF23: Fibroblast Growth Factor 23
AMBP: Protein Alpha-1-Microglobulin/Bikunin Precursor
CTSD: Cathepsin D
RAGE: Receptor For Advanced Glycosylation End Products
GRN: Granulins
CD93: Complement Component C1q Receptor
PAR-1: Proteinase Activated Receptor 1
SPON1: Spondin 1
ST2: Interleukin 1 Receptor-Like 1
FGF21: Fibroblast Growth Factor 21
CPA1: Carboxypeptidase 1
CCL15: C-C Motif Chemokine 15
IGFBP7: Insulin Like Growth Factor Binding Protein 7
IL1RL2: Interleukin 1 Receptor Like 2

Chapter 1

Introduction to Cardiovascular Disease Risk Assessment and Exploration of Novel Biomarkers

1.1 Introduction to Cardiovascular Disease

Cardiovascular disease (CVD) due to atherosclerosis of the arterial vessel wall was the leading cause of noncommunicable disease deaths worldwide in 2012 being responsible for 17.5 million deaths (46% of noncommunicable disease deaths). Of these deaths, an estimated 7.4 million were due to heart attacks and 6.7 million were due to strokes (1). CVD is divided into coronary artery disease (CAD), ischaemic stroke and peripheral arterial disease (PAD).

In Europe, CVD kills more than 4 million people each year. It kills more women [2.2 million (55%)] than men [1.8 million (45%)], although cardiovascular (CV) deaths before the age of 65 years are more common in men (490 000 deaths vs. 193 000 deaths) (2).

In the United Kingdom (UK), CVD was the second most common cause of death in 2014 causing 27% of all deaths, while cancer caused 29%. The Global Burden of Disease study has shown that the burden of CVD is declining in the UK (3). Nevertheless, CVD still constitutes a huge economic burden with £4.3 billion spent on treatment within the NHS in England in 2014 (4). Between 1979 and 2013, Northern Ireland had a 75% decrease in CVD mortality and a reduction of 87% for premature CAD mortality. However, there was still a 6% increase in hospital admissions for CAD in Northern Ireland between 2010/2011 and 2013/2014 (5). Despite large reductions in mortality from CVD, this condition has remained a substantial burden to the UK, with rises in hospital admissions for all CVD. The underlying pathological process behind the development and progression of CAD is atherosclerosis. The concept of atherosclerosis being a chronic inflammatory disease is being widely accepted nowadays, however there is still many underlying mechanisms yet to uncover.

1.1.1 Atherosclerosis

Atherosclerosis is a progressive build-up of cholesterol in the arterial wall accompanied by an endothelial dysfunction and an increased inflammatory response. This causes the artery to become narrow which reduces the blood flow and increases the risk of heart attack and clot formation.

1.1.1.1 Lipoprotein retention

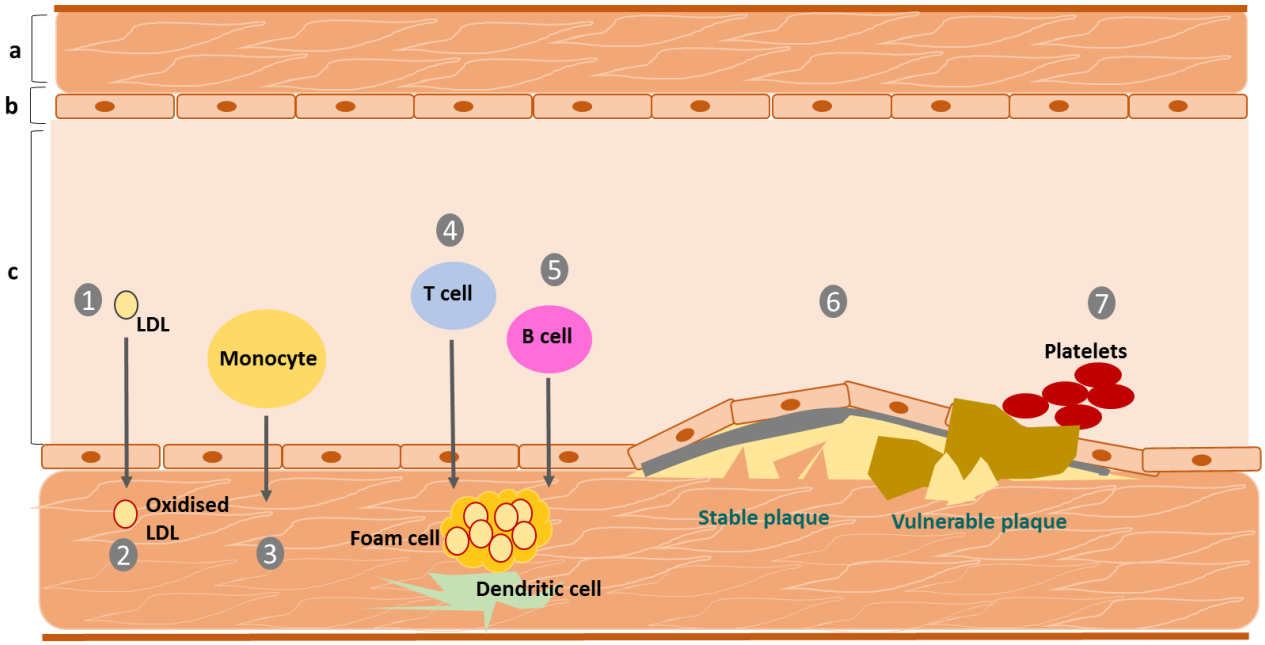
Atherosclerosis occurs in the intima of large and medium-sized arteries at regions of disturbed blood flow and is a result of an endothelial dysfunction, a subendothelial lipoprotein retention and a maladaptive immune response. Over time, atherosclerosis leads to a chronic inflammation in the arterial wall causing intimal destruction, arterial thrombosis and end-organ ischemia (6,7).

Atherosclerosis starts with a subendothelial retention of apolipoprotein B – containing lipoproteins (LPs) in particular regions of the arteries where laminar flow is disturbed by bends or branch points in the arteries (7,8).

1.1.1.2 Inflammatory response and fibrous cap formation

Different modifications of the retained LPs likely mimic pathogen- and/or damage-associated molecular patterns and subsequently trigger a low-grade inflammatory response. This response leads to activation of endothelial and vascular smooth muscle cells, recruitment of monocytes and accumulation of cellular, extracellular, and lipid material in the intima. The cells recruited to the inflammatory lesion include monocyte derived macrophages, T cells, B cells, dendritic cells, mast cells, and smooth muscle cells. In most of the cases, atherosclerotic lesions undergo a partial resolution process characterised by the formation of an overlying scar, or fibrous cap (9,10). This fibrous

cap forms a protective barrier between platelets in the blood stream and prothrombotic material in the plaque. In addition, outer remodelling of the arterial wall, resulting in the preservation of luminal blood flow and collateral vessel formation, help prevent end organ ischemia (Figure 1).



a. Vascular smooth muscle cells; b. Endothelial cells; c. Lumen; LDL: low density lipoprotein

***Figure 1*: Atherosclerotic plaque formation and progression.** *1. Atherosclerosis starts with a sub-endothelial retention of low density lipoprotein (LDL) particles in the arterial wall. 2. LDL particles undergo oxidation which 3. Triggers an inflammatory response with the recruitment of monocytes and foam cell formation. 4. This is followed by the recruitment of T cells, B cells, dendritic cells and smooth muscle cells. 5. This results in the formation of a fibrous cap in stable plaques. 6. Some plaques, known as vulnerable plaques, may undergo thinning of the fibrous cap and the subsequent formation of a necrotic core. 7. This leads to the exposition of thrombotic material to the blood circulation and the formation of thrombus.*

1.1.1.3 Progression of atherosclerotic lesions and vulnerable plaques

Most atherosclerotic lesions do not cause acute vascular disease (11), nevertheless, certain types of atherosclerotic lesions develop features over time that can result in an acute thrombotic vascular disease. These are known as “vulnerable plaques” and are characterised by a large area of necrosis in the intima, called the necrotic or lipid core, thinning of the fibrous cap and an increased inflammatory state. Fibrous cap thinning is

likely caused by a combination of defective collagen synthesis by intimal smooth muscle cells and an increased degradation by matrix metalloproteinases which are secreted by inflammatory cells. Activation of innate and adaptive immune pathways contribute to the inflammatory response, (12) and this is amplified in advanced lesions by the increased production of damage-associated molecular patterns (DAMP) from necrotic cells.

There are different ways that a vulnerable plaque can rupture. First, thinning of the fibrous cap leads to the exposure of thrombotic and proinflammatory material of the necrotic core to the circulating blood leading to thrombotic coronary occlusion. This type of plaque rupture is recognised to be the most common cause of myocardial infarction and death from cardiac causes (11). Second, the blood vessel can break leading to the formation of a thrombus on a cell-denuded endothelial layer of a non-ruptured plaque. Finally, calcified nodules are also reported to lead to clinical events and are considered as another type of vulnerable plaques (13).

To date, there is no reliable way to identify vulnerable plaques. Few techniques are currently used such as intravascular ultrasound (IVUS), which is an invasive coronary atherosclerotic plaque imaging technique, and optical coherence tomography. Nevertheless, apart from a proof of concept studies, none of them have successfully established criteria for assessing plaque vulnerability in a larger sense.

1.1.2 Likelihood of recurrences of cardiac events after first event

Nowadays, more patients are surviving their first heart attack but are nevertheless at high risk of recurrences. Recurrent events have also been referred to as major adverse cardiovascular events (MACE) in many studies and include death, myocardial infarction, stroke, heart failure and repeated coronary revascularisation (BCIS - British

Cardiovascular Intervention Society), (14,15). Even with access to the highest revascularisation strategy technology (Coronary Artery Bypass Grating (CABG) and Percutaneous Coronary Intervention (PCI)) as well as most recently available secondary prevention therapies, the burden of recurrent events after acute coronary syndromes remains unacceptable. The recurrences are of the order of 10% to 20% in the first 12 months, despite optimal treatment with contemporary intervention and pharmacological agents (16–18). MACE can be due to a recurrence at the original treatment site, the presence of untreated lesions elsewhere, or progressive lesions. Moreover, retrospective studies have shown that most atherosclerotic plaques responsible for future acute coronary syndromes are angiographically mild and the lesion-related risk factors for MACE are poorly understood (19).

After a heart attack, a number of measures are put in place in order to reduce the occurrence of MACE. As persistent high cholesterol levels (20) and high blood pressure (21) are known to be predictors of MACE, these factors are controlled using statins and anti-hypertensive therapy post infarction. Moreover, the patient is advised to modify his diet in order to control his body weight and practice regular exercise (22). Controlling diabetes when present has also proven to be a good way to avoid future MACE. However, despite these measures, MACE still occur (23).

Other risk factors have also been shown to predict MACE. In 2000, aortic stiffness was proposed to be an independent risk factor for recurrent acute coronary events (24). However, measuring aortic stiffness has shown to be insufficient as it is also part of a normal process present in older patients, patients with diabetes mellitus, hypertension and renal disease and might therefore need to be combined to other clinical measurements for an accurate prediction of MACE (25,26). In 2001, leukocyte count was reported as another marker of the proposed atherosclerotic inflammatory process that

might contribute to recurrent cardiac events in post-infarction patients (27). However, this is confounded by the fact that lymphocyte count can be elevated in many other inflammatory conditions which complicates its interpretation. Measuring platelet reactivity in *ex vivo* assays has been shown to be potentially useful in predicting MACE, however, these tests haven't made it into clinical routine practice (28). More recently, depression and anxiety have been linked to recurrent cardiac events (29). Since many variables can potentially influence risk, it seems unlikely that one mechanism is enough to effectively predict MACE.

The prediction of cardiovascular events has always focused on the occurrence of primary events (30) as Framingham Heart Study data and similar observational study data as well as the developed risk scores are used to predict vascular disease risk in people with no established CAD. Cardiovascular prediction research on the risk of recurrent events in patients with an established CAD also exists, however, there is no clinical tests at present that can predict MACE (31–33). Moreover, the number of young individuals with coronary atherosclerosis is probably much larger than currently estimated (34) and this young population is often less represented in clinical studies (35). Multivariable analysis of MACE risk could provide an individualised risk scoring system and help identify individuals at higher risk for more intensive investigation, treatment, and follow-up.

1.2 Acute coronary syndrome (ACS) and myocardial infarction *(ESC guidelines 2015)*

1.2.1 Definition and diagnostic algorithm

Acute coronary syndromes (ACS) are recognised as medical emergencies that include unstable angina (UA), non-ST-segment-elevation myocardial infarction (NSTEMI) and ST-

segment-elevation myocardial infarction (STEMI). A myocardial infarction is defined as a myocardial cell necrosis due to significant and sustained ischaemia (36) (WHO). It is the result of a reduction of blood flow to the heart usually caused by plaque rupture which can sometimes lead to the formation of blood clot (NICE guidelines). According to the European Society of Cardiology (ESC) guidelines, diagnosing an ACS follows the algorithm below (Figure 2):

1- Clinical symptoms include acute chest pain characterised by a retrosternal sensation of pressure or heaviness ('angina') radiating to the left arm (less frequently to both arms or to the right arm), neck or jaw, which may be intermittent (usually lasting several minutes) or persistent. Additional symptoms such as sweating, nausea, abdominal pain, dyspnoea and syncope may also be present.

2- An emergency electrocardiogram (ECG) analysis is performed in order to identify:

   a. **Patients with acute chest pain persistent ST -segment elevation** generally reflecting a total coronary occlusion and could ultimately lead to the development of an ST-elevation myocardial infarction **(STEMI)** (37)**.**

   b. **Patients with acute chest pain but no persistent ST-segment elevation.** At presentation, the working diagnosis of non-ST-elevation ACS **(NSTE-ACS)**

   c. Based on the measurement of cardiac troponins with at least one value above the 99th percentile of the upper reference limit, NSTE-

ACS will be further qualified as non-ST-elevation MI **(NSTEMI)** if cardiac troponins are elevated or unstable angina **(UA)** if they are not.

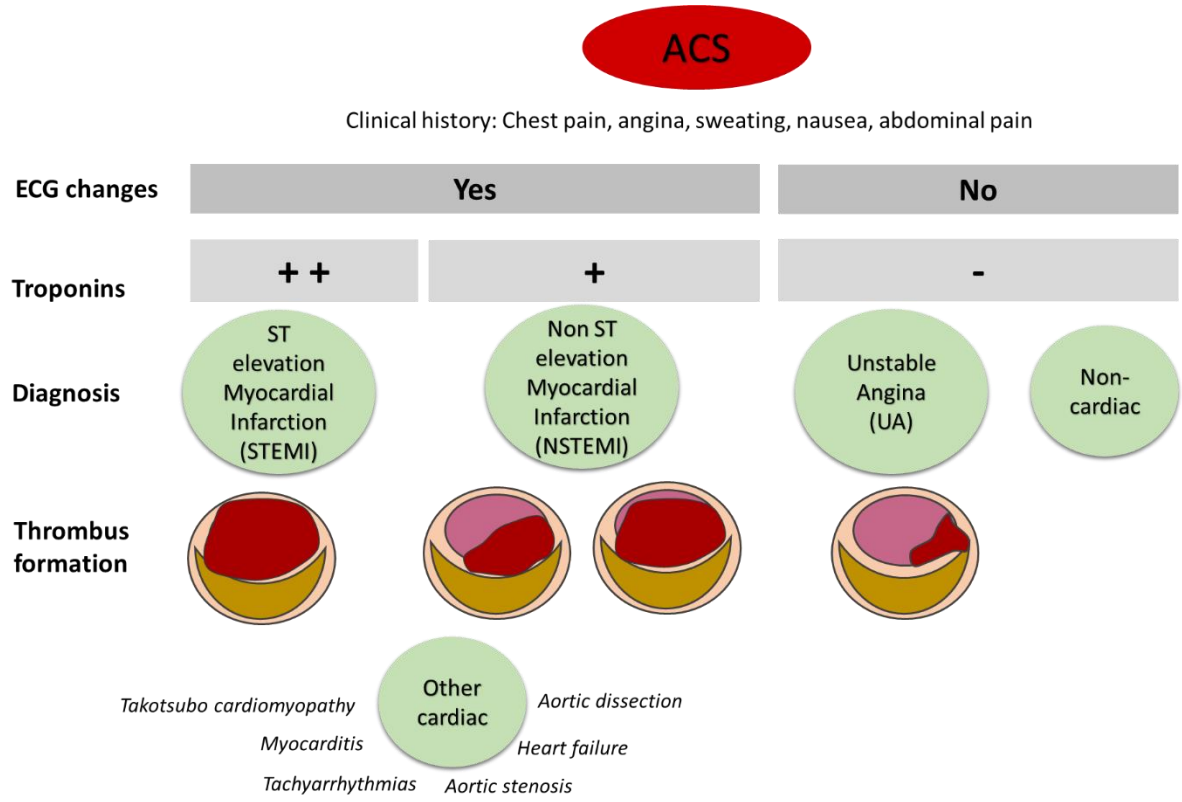


***Figure 2**: **Initial assessment of patients with suspected Acute Coronary Syndrome (ACS).** The initial assessment of patients with suspected ACS is based on the features derived from clinical presentation. A 12-lead ECG is then performed to assess any changes in the heart rhythm and electrical activity. A cardiac troponin test is performed and levels are interpreted as a quantitative marker: the higher the level, the higher the likelihood for the presence of myocardial infarction. The presence of a severe thrombus is associated with a higher chance of having a STEMI. ECG changes and elevated cardiac markers can be present in other cardiac conditions such as Takotsubo cardiomyopathy, myocarditis, aortic dissection and heart failure. Adapted with minor modifications from 2015 ESC Guidelines for the management of acute coronary syndromes in patients resenting without persistent ST-segment elevation* (38).

In addition to the traditional CVD risk factors mentioned in **section 1.3.3**, many conditions are known to precipitate an ACS such as intense emotions, intense exercise, cold temperatures, anaemia, infection, fever, inflammation and metabolic disorders (38). In addition, imaging evidence of new or presumed new loss of viable myocardium or regional wall motion abnormality as well as intracoronary thrombus detected on angiography or autopsy are also considered as ways to confirm an MI.

1.2.2 STEMI *versus* NSTEMI

A STEMI is considered to be more severe than a NSTEMI because mortality is generally higher in the STEMI group (39). A NSTEMI occurs by developing a complete occlusion of a minor coronary artery or a partial occlusion of a major coronary artery affected previously by atherosclerosis. This causes partial thickness damage of heart muscle historically referred to as a non-Q wave MI or a subendocardial MI. On the other hand, a STEMI occurs by developing a complete occlusion of a major coronary artery previously affected by atherosclerosis (Figure 2). This causes full thickness damage of heart muscle historically referred to as Q wave MI or transmural MI. Also, the elevation of cardiac markers is on average lower in a NSTEMI compared to a STEMI (40,41). The incidence of STEMI appears to be declining, while there is a concomitant increase in the incidence of NSTEMI (42). This is probably because the troponin measurement assays are increasing in sensitivity and specificity which makes it possible to detect a NSTEMI earlier leading to the initiation of a proper therapy before the development of a STEMI and other complications (43).

1.2.3 Unstable angina

Unstable angina (UA) is defined as myocardial ischaemia at rest or minimal exertion in the absence of myocardial cell necrosis. Compared with NSTEMI patients, individuals with UA do not experience myocardial necrosis that is currently detectable with the clinical available biomarkers, have a substantially lower risk of death and appear to have less benefit from intensified antiplatelet therapy as well as early invasive strategy (44–47).

1.2.4 Cardiac troponins

Troponin I (Tn I) and troponin T (Tn T) are cardiac proteins involved in the regulation of muscle contraction by controlling the calcium mediated interaction between actin and myosin. Tn I has not been identified outside the heart muscle (48) whereas Tn T can also be secreted by the skeletal muscle. Tn I and Tn T are detected in the serum using monoclonal antibodies which have high specificity to the cardiac troponins and negligible cross-reactivity with skeletal troponins (49). Cardiac troponins may not be detected for up to 4 hours after the onset of an ACS and are normally repeated after 12 hours if levels were not elevated upon admission (50). Tn T assay is measured using a single assay so that levels can be compared across laboratories with a cut-off value of 0.1 µg/litre. However, Tn I is measured using different assays with different sensitivities (51) and the European Society of Cardiology (ESC) and American College of Cardiology (ACC) consensus recommends that each laboratory should determine its cut-off values for each test at the 99th centile of normal with ≤ 10% coefficient of variation (52) (Refer to **section 1.4.4**). In line with these recommendations, serum Tn I values indicative of myocyte necrosis/myocardial damage range from 0.1 to 2 µg/litre (53) . The sensitivity of Tn T and Tn I assays improve with time following admission with a maximal sensitivity at 6 hours after the onset of an ACS (50). Serum levels can remain elevated for up to 4–7 days for Tn I, and 10–14 days for Tn T (54).

While troponin is helpful in the diagnosis of ACS, it lacks specificity and its clinical use is more effective in ruling out patients with no heart attack but, less effective in ruling in patients with a heart attack as Tn I levels can be elevated for other reasons detailed in Table 1.

Table 1: **Conditions with elevated troponins**

Tachyarrhythmias
Heart failure
Hypertensive emergencies
Critical illness (e.g. shock/ sepsis/ burns)
Myocarditis
Takotsubo cardiomyopathy
Structural heart disease (e.g. aortic stenosis)
Aortic dissection
Pulmonary embolism, pulmonary hypertension
Renal dysfunction and associated cardiac disease
Coronary spasm
Acute neurological event (e.g. stroke or subarachnoid haemorrhage)
Cardiac contusion or cardiac procedures (CABG, PCI, ablation, pacing, cardioversion, or endomyocardial biopsy)
Hypo- and hyperthyroidism
Infiltrative disease (e.g. amyloidosis, haemochromatosis, sarcoidosis, scleroderma)
Myocardial drug toxicity or poisoning (e.g. doxorubicin, 5-fluorouracil, herceptin, snake venoms)
Extreme endurance efforts
Rhabdomyolysis

Adapted with courtesy of the 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation (38)

1.3 Risk of cardiovascular disease

1.3.1 Defining risk

A risk factor is defined as a measurable characteristic associated with increased disease frequency (55). According to the European Society of Cardiology (ESC) guidelines, risk estimation in cardiovascular disease is defined as the 'likelihood of a person developing an atherosclerotic cardiovascular event over a period of time'. Calculating and interpreting a patient's cardiovascular risk is generally followed by several preventive lifestyle and medical measures where both the physician and the patient have a major role to play in. Clinical thresholds, which are based on the results of large clinical studies, are used in practice order to trigger intervention.

1.3.2 History of risk

A century of intensive research has shown that the presence of CVD is attributed to genetic, social, physiological, and environmental factors. Cardiovascular epidemiology began around the 1950's when several epidemiological studies were set up with the aim of clarifying why patients get CVD. Four years after the Framingham Heart Study started, researchers had identified high cholesterol and high blood pressure levels as important factors in the development of CVD (55). In a follow up study that started in 1983, obesity was subsequently considered as an independent risk factor for CVD (56). Further down the line, other epidemiological studies have helped with identifying other CVD risk factors such as diabetes (57), dietary composition and fibre consumption (58) as well as kidney disease (59).

1.3.3 Traditional risk factors

The five major traditional risk factors known to be associated with CVD are total cholesterol levels, systolic blood pressure (SBP), tobacco smoking, diabetes and family history. These factors are included in most of the risk prediction scoring systems. Hypertension is defined as a systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg. These cut-off blood pressure (BP) values are based on evidence from randomised control trials where treatment-induced BP reductions were beneficial in patients with these cut-off BP values (60). As for total cholesterol levels, the threshold for intervention is set at ≥ 4.9 mmol/L or 190 mg/dL as per the ESC guidelines for a patient with no other CVD risk factors. However, if the patient has a number of CVD risk factors (such as the presences of a family history, diabetes, or smoking) the threshold in terms of total cholesterol levels for clinical intervention is lower (61).

Other risk factors known to contribute to cardiovascular risk include renal disease (62), lack of physical activity, dietary habits and obesity (63), (world health federation, risk factors, 2015). Nevertheless, it is well established that traditional risk factors account for only 50% of the incidence of CVD (64). In fact, many individuals who develop heart disease have normal cholesterol and blood pressure levels (64) which raises the point that these traditional risk factors do not fully explain CVD risk. Many physiological and social risk factors have recently been shown to contribute to CVD risk beyond the traditional CVD risk factors (Table 2). This highlights the need for a broader approach when it comes to risk estimation.

Table 2: **Newly proposed risk factors for cardiovascular disease**

Risk Factor	Association with CVD	Studies
Atrial fibrillation	Risk factor for cardiovascular disease and death in women compared with men	(65)
Migraine	Increased risk of cardiovascular disease in women	(66)
Ankylosing spondylitis	Prevalent patients with ankylosing spondylitis are at a 30%–50% increased risk of incident CV events	(67)
Homelessness	Independent Risk Factor for Cardiovascular Disease Hospital Readmission	(68)
Anxiety	Important risk factor for cardiovascular disease and increases the risk of major cardiac events in coronary heart disease	(69)
Depression	Increased risk for CVD in middle-aged women in the general population	(70)
Loneliness and social isolation	Deficiencies in social relationships are associated with an increased risk of developing CAD and stroke	(71)
Circadian misalignment (shift work)	Increases hypertension, inflammation, and cardiovascular disease risk through an increase in blood pressure and inflammatory markers	(72)
Bowel Movement Frequency	Compared to those with daily bowel movements, women with more frequent bowel movements had a modestly increased risk of CAD incidence and total mortality	(73)
Sleep apnea	Independent risk factor for cardiovascular disease among professional drivers	(74)
Consumption of sweetened beverages with high-fructose corn syrup	The risk of cardiovascular mortality is positively associated with consumption of increasing amounts of added sugars	(75)

CVD: Cardiovascular disease, CAD: Coronary artery disease, CV: Cardiovascular

1.3.4 Risk prevention

Age-adjusted CVD mortality has declined since the 1980s, particularly in high-income regions (76) due to the implementation of preventive measures. CVD rates are now less than half what they were in the early 1980s in many countries in Europe. In fact, in the last three decades, more than half of the reduction in CV mortality has been attributed to modification of risk factors in the population, primarily by reducing cholesterol, BP and smoking. While CVD management is improving, the number of people affected by CVD is increasing due to the increase in other risk factors, mainly obesity and type 2 diabetes mellitus (77,78). Changes in lifestyle behaviours such as smoking, physical activity and dietary habits would make it possible to prevent at least 80% of CVD and 40% of cancers, thus providing added value for other chronic diseases (79,80). Ultimately, prevention is effective in reducing the impact of CVD but further work is needed to explore this.

1.3.5 Risk scores

Since CVD is the product of a multitude of risk factors, all current guidelines recommend the assessment of total CVD risk. Therefore, CVD prevention in an individual should be adapted to his/her total cardiovascular risk; the higher the risk, the more intense the action should be. Most guidelines use risk estimation systems based on the most popular risk estimation scores which are the Framingham Risk Score or the European SCORE projects and are highlighted below and in Table 3. Risk estimation can be determined either for primary or secondary prevention. Primary prevention is established to control CVD risk factors in patients with no prior CVD history in order to delay or prevent the onset of CVD (81). In contrast, secondary prevention is established

to control CVD risk factors in patients with established CVD in order to prevent the occurrence of further cardiovascular events (82).

1.3.5.1 Risk Scores used in primary prevention

1.3.5.1.1 EUROSCORE (European Systematic COronary Risk Evaluation (SCORE) algorithm)

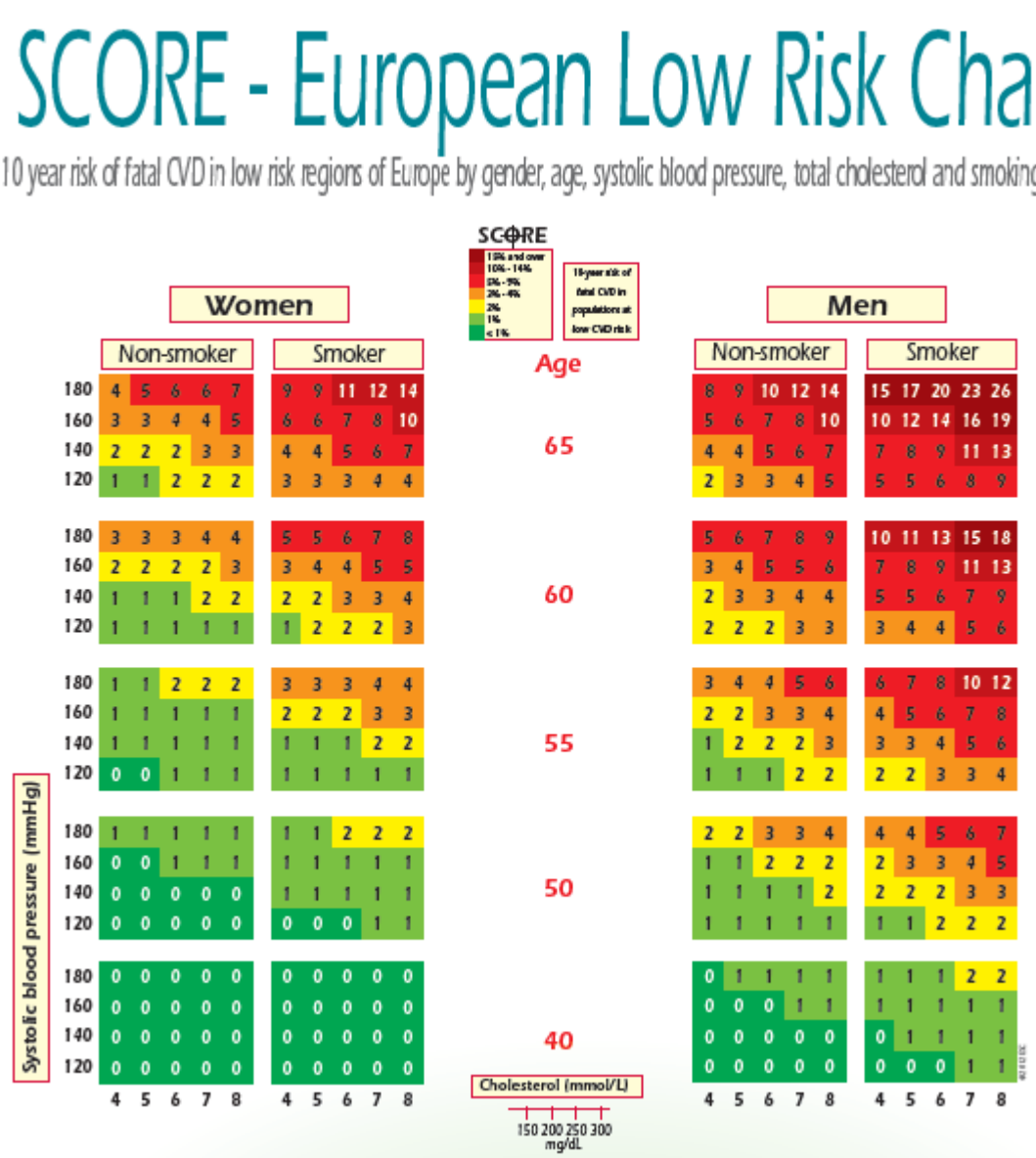
In Europe, the SCORE system is used in primary prevention to assess an individual's cardiovascular risk. This SCORE system was established based on studies in different European cohorts and using other scoring systems won't be as accurate to establish the cardiovascular risk of a European individual due to genetic background (Figure 3 and Table 3). The development of the SCORE system by the European Society of Cardiology was initiated between 1998 and 2001 using data from 12 European cohort studies (n=205,178) covering a wide geographic spread of countries with different levels of cardiovascular risk (83). This resulted in the generation of two SCORE charts depending on where an individual comes from which means they can fall into a low or high CVD risk region in Europe. The SCORE data contains more than 3-million person-years of observation and 7,934 fatal cardiovascular events. It estimates the 10-year risk of a first fatal atherosclerotic event, whether heart attack, stroke, or other occlusive arterial disease, including sudden cardiac death.

In the case of primary prevention defined as the control of CVD risk factors to delay or prevent the onset of CVD, risk estimation is calculated using SCORE charts which are based on the following risk factors: gender, age, smoking, systolic blood pressure and total cholesterol. The threshold for high risk based on 10-year risk fatal cardiovascular events is defined as "higher than 5%". To calculate risk the above variables are inputted into the appropriate score chart and then the risk calculated. This has become extremely

easy with the development of the smart phone applications and online calculators. Patients with diabetes or moderate to severe chronic kidney disease are immediately classified as very high risk and so, SCORE charts do not apply.

Subsequent work on the SCORE database has shown that high density lipoprotein cholesterol (HDL-C) can contribute greatly to risk estimation if entered as a separate variable and was shown to modify risk at all levels of risk as estimated from the SCORE with the same effect in both genders and in all age groups (84,85). That is the reason behind the development of the heartScore chart which takes into account the HDL-C level to score patients (http://www.heartscore.org/).

On the other hand, fasting triglyceride (TG) levels related to risk in univariate analyses, but the effect is diminished by adjustment for other factors, especially HDL-C. Non-fasting TG have been suggested to be more strongly related to risk independently of the effects of HDL-C (86). The effect of additional risk factors such as high sensitivity C-reactive protein (hs-CRP) and homocysteine levels was also considered. However, the contribution of these risk factors to absolute cardiovascular risk estimations for individual patients (in addition to the older risk factors) is generally considered to be modest.



<u>Figure 3:</u> EUROSCORE European low risk chart for the assessment the 10-year risk of fatal CVD in low risk regions of Europe. *The use of the low risk chart is recommended in Andorra, Austria, Belgium, Cyprus, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Israel, Italy, Luxemburg, Malta, Monaco, The Netherlands, Norway, Portugal, San Marino, Slovenia, Spain, Sweden, Switzerland and the United Kingdom. The use of the high-risk chart is recommended in other European countries.*

1.3.5.1.2 Other Risk Scores used in primary prevention

There are several other scoring systems in place that assess cardiovascular risk. In the United States, the use of the Framingham risk score (FRS) is more common. This scoring system estimates the 10-year cardiovascular risk and was developed based on the data from the Framingham heart study, which recruited 5,209 men and women, to estimate the risk of developing coronary heart disease (Framingham study website.org). Moreover, the Prospective Cardiovascular Münster (PROCAM) study was initiated in 1979 and its objectives were to determine the prevalence of CAD risk factors in the German population, improve the prediction and early detection of CAD, and derive recommendations for the primary prevention of vascular disease from the trial results. The PROCAM score resulting from this study is also used to assess cardiovascular risk. QRISK is another scoring system available to calculate the 10-year risk of CV events and it was established using a sample size of 1.28 (QRISK1) and 2.29 (QRISK2) million people.

A study compared the FRS, PROCAM and SCORE ability to predict coronary atherosclerosis and cardiovascular events. The results showed that in a stable chest pain population, the ability of FRS and SCORE to predict CAD was similar and better compared to PROCAM. However, the number of low risk patients showing significant CAD or events was lower using FRS (87). However, more efforts should be done towards the encouragement of the use of any of those risk charts in clinical practice since many patients with high blood pressure or high cholesterol levels or any of the previously mentioned risk factors are not detected and treated on time before they develop an ACS (88).

Recent work has proposed a new scoring system predicting cardiovascular risk worldwide which is based on the fact that a higher percentage of people are at high risk

of fatal CVD in low and middle income countries rather than in higher income countries (89). However, the validation of this tool in clinical practice needs to be assessed in larger cohorts.

***Table 3**: Current cardiovascular disease risk estimation systems for use in apparently healthy individuals.*

	Data	**Population and size**	**Outcome**	**Variables**	**Recommended in Guidelines**	**Reference**
Framingham	Prospective studies: Framingham Heart Study and Framingham offspring study. Latest version includes Both (USA)	General population, Framingham, Mass, U.S. Volunteer, Baselines: 1968–1971, 1971–1975, 1984–1987 3969 men and 4522 women	10-year risk of CAD events originally. Latest version: 10-year risk of CVD events NCEP ATP III version: 10-year risk of hard coronary events	Sex, age, total cholesterol, HDL-C, SBP, smoking status, DM, hypertensive treatment	NCEP guidelines, Canadian CV guidelines, other national guidelines recommend adapted versions including New Zealand	(90)
SCORE	12 pooled prospective Studies from 11 European countries (Europe)	Mostly random samples from general population, some occupational cohorts, baselines: 1972–1991 117 098 men and 88 080 women	10-year risk of CVD mortality	Sex, age, total cholesterol or total cholesterol/ HDL-C ratio, SBP, smoking status. Versions for use in high and low-risk countries	European Guidelines on CVD Prevention	(83)
ASSIGN SCORE	SHHEC prospective study (Scotland)	Random sample from general population in Scotland, baseline: 1984–1987	10-year risk of CVD events	Sex, age, total cholesterol, HDL-C, SBP, smoking – no.	SIGN (Scottish Intercollegiate Guidelines Network)	(91)

		6540 men and 6757 women		cigs, DM, area based index of deprivation, family history		
QRISK1 and QRISK2	QRESEARCH database (UK)	Health records of general practice attendees - not random, baseline: 1993–2008 1.28 million (QRISK1) 2.29 million (QRISK2)	10-year risk of CVD events. Lifetime risk	QRISK1 - sex, age, total cholesterol to HDL-C ratio, SBP, smoking status, DM, area based index of deprivation, family history, BMI and BP treatment QRISK2— also includes ethnicity and chronic diseases	NICE guidelines on lipid modification, QRISK Lifetime recommended by JBS3 guidelines	(92) (93)
PROCAM	Prospective study (Germany)	Healthy employees. Baseline: 1978–1995 18 460 men and 8515 women	Two separate scores calculate 10-year risks of major coronary events and cerebral ischaemic events	Age, sex, LDL-C, HDL-C, DM, smoking, SBP	International Task Force for Prevention of Coronary Disease Guidelines	(94)

ATP = Adult Treatment Panel; BMI = body mass index; BP = blood pressure; CAD = coronary artery disease; CVD = cardiovascular disease; DM = diabetes mellitus; HDL-C = high-density lipoprotein cholesterol; JBS = Joint British Societies; LDL-C = low-density lipoprotein cholesterol; NCEP = National Cholesterol Education Program; NICE = National Institute for Health and Care Excellence; no. cigs = number of cigarettes; PROCAM = Prospective Cardiovascular Munster Study; SBP = systolic blood pressure; SIGN = Scottish Intercollegiate Guidelines Network; SHHEC = Scottish Heart Health Extended Cohort.

1.3.5.1.3 Weaknesses of the currently available scoring systems used in primary prevention

Despite the advantages of having risk estimation tools in place to guide therapeutical strategy and patient management, they still hold many weaknesses and might need to be combined to other biological tests to improve cardiovascular risk estimation. Several highlighted problems with the current scoring systems are detailed below.

1.3.5.1.3.1 Effect of one risk factor at different levels of other risk factors

One limitation of all risk estimation systems is that they assume constant effects of the risk factors at differing ages and levels of the other risk factors. Certain combinations of risk factors may act synergistically to increase risk in a manner that is more than additive. The ideal solution for this problem would be to have an extremely large dataset (a whole country or even a continent) in which there were numerous persons with each combination of risk factors and to examine the actual risk within each combination. In this method, particularly dangerous combinations of risk factors could be identified. However, development of such a dataset would be practically impossible, especially in the modern era when many of the identified risk factors already have been treated (95), therefore, alternative methods should be investigated.

1.3.5.1.3.2 Recalibration of the scoring systems according to the different regions

Risk functions developed in one region will tend to overestimate or underestimate CVD risk in other populations with different baseline risks, either because of changes over time or regional differences. For example, the use of the high risk SCORE risk chart in very high risk regions of Europe could underestimate the risk in countries such as

Bulgaria, Latvia and Lithuania. This highlights the need to update the existing scoring systems based on recent prospective studies, the current CVD mortality rates and the different levels of risk factors in a population of a specific region (96).

1.3.5.1.3.3 Evaluating the introduction of new risk factors to the scoring systems

As mentioned above, traditional risk factors included in most of the scoring systems account for only 50% of the incidence of CVD (64). Adding “new” risk factors to the traditional CVD risk factors might help to appropriately reclassify some of the patients who are close to a treatment threshold to a more correct “treat/do not treat” category (95). Assessing the value of incorporating new risk factors into risk estimation systems has been traditionally evaluated by the Area Under the Receiving Operating Characteristic (AUROC) curve or Harrell's C statistic. However, AUROC was a technique developed for assessing the performance of a diagnostic test that has a straightforward yes/no answer, against that of a gold standard. A perfectly sensitive and specific test will result in an AUROC of 1. However, because risk estimation is just an estimate, a perfect AUROC could never be achieved and its use is more appropriate in diagnostic tests rather than in risk estimation. For this reason, there has been increasing interest in developing more appropriate methods to judge the improvement in performance induced by the incorporation of new risk factors (97). The method with most potential for clinical utility is patient reclassification and discrimination and calculation of measurements such as c statistics, the net reclassification improvement (NRI) index and the integrated discrimination improvement (IDI) index (98,99).

It is noteworthy that when considering variables to include in a model for the generation of a risk score, selecting factors that report on orthogonal aspects of pathogenesis is important. For example, a factor for lipid risk, such as low-density

lipoprotein, myocardial stress, such as a natriuretic peptide, myocardial injury, such as troponin, inflammation, such as high sensitivity C-reactive protein, and glycemia, such as hemoglobin A1c, each report on different biological pathways (100–103). Inclusion of factors that lie in a common pathway (eg, low-density lipoprotein cholesterol, apolipoprotein B, and non–high-density lipoprotein cholesterol) would not be expected to add as much information to a risk assessment compared to factors that reflect orthogonal pathogenic pathways.

1.3.5.1.3.4 Reassessing the risk in young and old individuals

Younger individuals will always be at low absolute risk even when risk factor levels are very unfavourable, unless they suffer from a genetic defect that results in higher risk such as homozygous familial hypercholesterolemia. When looking at the 40-year age band of the SCORE chart for example, no combination of risk factors will place a person in the high-risk category (≥5% 10-year risk of fatal CVD). Even a 40-year-old man who is severely hypertensive, severely hypercholesterolemic, and a smoker will still only have a risk of 4%. The same situation occurs with use of the Framingham function. This represents a challenge when counselling these younger persons regarding the need for lifestyle modification to reduce their risk (104). This is an important issue since the modification of risk factors at this early stage has a great benefit on CVD prevention. The relative risk chart has been developed in the Fourth Joint Task Force ESC guidelines to counteract this problem which provides an estimate of the risk of a person with a certain combination of risk factors compared with a person of the same age and sex who has ideal risk factor levels.

Similarly, the estimation of risk in the elderly remains a challenge. In some age categories included in the SCORE system, the clear majority, especially men, will have

an estimated cardiovascular death risk exceeding the 5– 10%, based on age (and gender) only, even when other cardiovascular risk factor levels are relatively low. This could lead to excessive usage of drugs in the elderly and should be evaluated carefully by the clinician. Furthermore, the SCORE function concentrates on the middle-aged group and is only recommended for use in the 40- to 65-year age range (83) which makes risk estimation between the ages of 65 and 75 years problematic as most of these systems were derived from cohorts of primarily middle-aged people.

1.3.5.2 Risk Scores used in secondary prevention

1.3.5.2.1 Current risk scores

Patients who have had a clinical event such as an ACS or a stroke or a clinical intervention such as a PCI or a CABG fall into the category of secondary prevention. In this case, we estimate the risk of further cardiovascular events. The GRACE risk score was based on an international registry designed to track in-hospital and long-term outcomes of patients presenting with ACS (105–107). The GRACE 2.0 risk calculator (Figure 4) provides a direct estimation of mortality while in hospital, at 6 months, at 1 year and at 3 years. The combined risk of death or MI at 1 year is also provided (108). Variables used in the GRACE 2.0 risk calculation include age, systolic blood pressure, pulse rate, serum creatinine, Killip class at presentation, cardiac arrest at admission, elevated cardiac biomarkers and ST deviation.

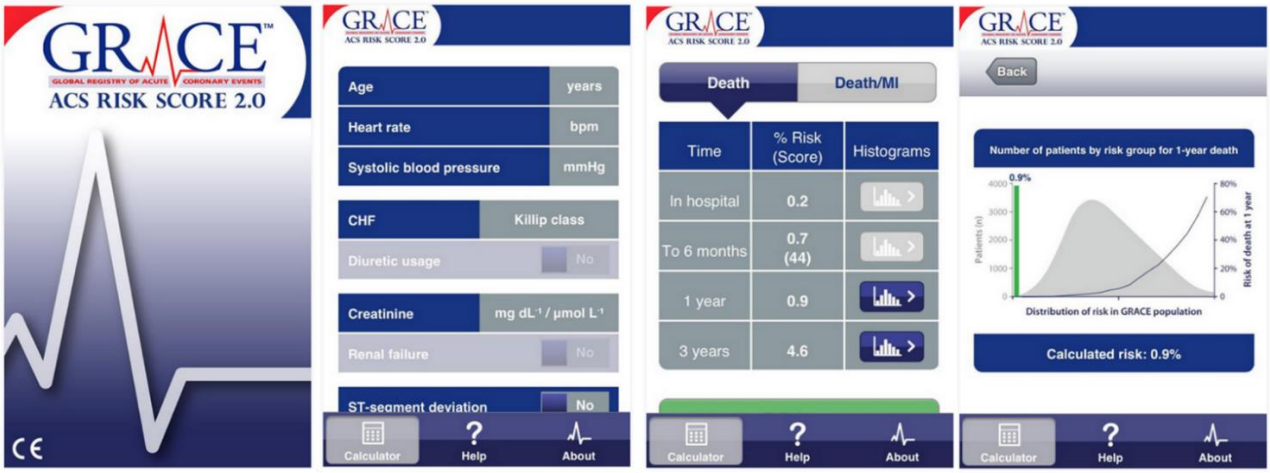


***Figure 4:* GRACE 2.0 risk score calculator.**

Moreover, the TIMI risk score uses seven variables in an additive scoring system: age ≥65 years, three or more CAD risk factors, known CAD, aspirin use in the past 7 days, severe angina (two or more episodes within 24 h), ST change ≥0.5 mm and positive cardiac marker (http://www.timi.org/index.php?page=calculators). TIMI risk score is known to be simple to use, but its discriminative accuracy is inferior to that of the GRACE risk score and the GRACE 2.0 risk calculation. PURSUIT and FRISC risk scores have also been developed and validated in patients with confirmed acute coronary syndrome (109). Further cardiovascular risk scores in specific disease conditions (diabetes, renal disease, rheumatoid arthritis, and obesity) also exist. However, ACS prediction models need to be re-evaluated in contemporary practice with evolving diagnostic and treatment options.

1.3.5.2.2 Limitations of the Risk Scores used in Secondary Prevention

Most of the available risk scores used in secondary prevention are only applicable to patients with an ACS and might not be as effective in patients with established CVD but with no previous cardiovascular events. In practice, it was shown that only a limited number of ACS patients actually have their GRACE score calculated (110) probably due to a lack of awareness by medical and nursing staff and a lack of time especially since

the creatinine and the troponins measurements are required for the score calculation. In addition, GRACE score has been criticised because the age variable makes a major contribution to the score calculation and might lead to an underestimation of the risk in younger CVD patients.

On the other hand, the TIMI risk score gives one point for every variable included in the model which assumes that all variables have the same impact on risk estimation. For example, the effect of using aspirin in the past 7 days is considered to have the same value as having more than 3 CVD risk factors or positive troponin levels. This is misleading as some variables might contribute more than others to the occurrence of secondary events. Ultimately, whether risk factor scores are used in primary or secondary prevention, it is essential to update them frequently according to the results of the latest clinical prospective studies and the discoveries in terms of novel CVD risk factors.

1.3.5.4 The need for more effective risk estimation models

Existing scoring systems should be improved given the weaknesses discussed previously. A significant proportion of individuals who have an MI are still categorised as low risk by many of the available methods. The current risk scoring systems for primary and secondary prevention need constant update and refinement based on current prospective cohort studies and any recent discoveries in relation to novel cardiac risk factors and biomarkers. In addition, risk estimation in a young or an old individual needs to be refined.

Since it is well-established that atherosclerosis is an inflammatory disease, some inflammatory pathways are more important in the initiation of the lesion and others are more prominent when the plaque is more likely to rupture. Measuring a combination of

inflammatory markers, specific for each pathway, will give a clearer image of the underlying state of the disease and the likelihood of the occurrence or recurrence of a cardiovascular event. Biomarkers are powerful tools that could improve the current CVD risk estimation models by looking at those different inflammatory pathways. By combining novel biomarkers to the traditional risk assessment scores, it would be possible to detect individuals which risk has been over or underestimated when using the traditional risk scores alone. Additionally, it will also be possible to identify individuals at high risk of recurrent events.

1.4 Biomarkers

Traditional risk assessment tools for primary or secondary prevention in CVD have several limitations. However, they can be improved by the addition of biomarkers that reflect the underlying inflammatory state of CVD.

1.4.1 Biomarker definition

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention, a definition that was standardised in 2001 by the National Institutes of Health (NIH) working group. Biomarkers are indicators of disease trait (risk factor or risk marker), disease state (preclinical or clinical), or disease rate (progression) (111). A biomarker can be a gene mutation, a polymorphism, a protein, or other molecule or clinical measurement that indicates a given disease state. Therefore, biomarkers could be measured in a biosample such as a blood, urine, or tissue test, recorded or obtained from a person (blood pressure, ECG, or Holter

monitor), or from an imaging test (echocardiogram or CT scan) (112). Biomarkers can be classified as:

1- Antecedent biomarkers: Identify the risk of developing an illness

2- Screening biomarkers: Screen for subclinical disease

3- Diagnostic biomarkers: Recognise the presence of a disease

4- Staging biomarkers: Categorise disease severity

5- Prognostic biomarkers: Predict future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy

1.4.2 Ideal biomarker

The ideal biomarker will increase the ability of the clinician to optimally manage the patient and will allow to distinguish between sub categories of patients that require a different type of management, a concept also known as personalised medicine (9). It is also important to examine the additional value of any novel biomarker to the existing and well-established risk factors. Ideally, a new biomarker should not only provide independent information on cardiovascular risk, but should also be easy to measure using inexpensive and standardised commercial assays with low variability that do not require specialised plasma collection or complicated assay techniques (9).

1.4.2.1 Characteristics of a biomarker

A novel biomarker would have added clinical value if it is: (113)

1- Accurate, which refers to the degree of agreement with a reference standard for the analyte and is quantified in terms of percent bias (Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS). Harmonized terminology database: 2005).

2- Precise, which refers to consistent measurement on replicates (Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS). Harmonized terminology database: 2005). One of the ways to evaluate precision is by measuring the coefficient of variation (CV) which is the standard deviation of a set of values divided by the average expressed in percentage.

3- Acceptable to the patient

4- Produces reproducible results in a standardised manner

5- Highly sensitive and specific in relation to the outcome. Sensitivity is defined as the probability of getting a positive test result in subjects with the disease. Whereas specificity is defined as a proportion of subjects without the disease with negative test result in total of subjects without disease (114). These are normally expressed in terms of receiver operating characteristics (ROC) curve. Analytical sensitivity of an assay is related to two measurements. First, the limit of detection (LoD) which is the smallest amount or concentration of analyte that can be distinguished from background at a stated confidence level. Second, the limit of quantitation (LoQ) which is the lowest amount of analyte in a sample that can be quantitatively determined with stated acceptable precision and accuracy, under stated experimental conditions (115).

6- Easily interpreted by the clinicians

7- It explains a reasonable proportion of the outcome independently of established predictors

8- There is enough knowledge linking biomarker levels and changes in patient's management

9- Tested in a large spectrum of people with varying degrees of pathology

10- Internationally standardised

However, these characteristics could change according to the intended use of each biomarker (116):

- **Screening biomarkers to identify "vulnerable patients":** High sensitivity, specificity, and predictive values, large likelihood ratios, and low costs are more important.

- Diagnostic markers of acute cardiac disease identifying ischemia or injury (such as acute MI): In addition to the aforementioned characteristics, high tissue specificity (referring to a myocardial origin), rapid sustained elevation, proportional release to the extent of the disease, assay features applicable to point-of-care testing and diagnostic cut-off well defined and accepted are critical (117).

- Biomarkers monitoring disease progression or response to therapy: In that case, sensitivity and specificity are less important features as each patient serves as his/her own control, but small intra-individual variation and changes in levels with disease outcome or treatment are important.

Additionally, some biomarkers (eg, exercise stress test) may be used for both diagnostic and prognostic purposes.

1.4.2.2 Assessing the value of a biomarker

The prognostic utility of a biomarker is more challenging to establish because it requires a large number of patients and a prospective design, whereas demonstrating its value as a diagnostic test requires a smaller sample and a cross-sectional design (118) which is normally done in feasibility studies. The value of a new biomarker is demonstrated when elevated risk of an outcome is associated with higher levels of the new biomarker with adjustment for established risk factors. These results are typically presented as hazards ratios (relative risk estimates from a Cox model) and a probability value test of significance of the marker in multivariable models.

It is noteworthy to mention that biomarkers that do not change disease management cannot affect patient outcome and are less likely to be useful. Therefore, the biomarker needs to prove its clinical utility by providing evidence that a strategy reducing risk will change depending on the biomarker levels. In order to assess this, biomarker levels need to be measured over time and interpreted according to any adverse event that might occur. This suggests that biomarker levels should be directly or indirectly modifiable by therapy (119). In summary, using a biomarker-guided approach has to lead to better patient outcomes compared to an approach without the use of the biomarker.

1.4.3 Single *versus* multiple biomarkers

While the idea of using a single marker seems clinically appealing due to simplicity and low cost, it is becoming more and more apparent that CVD is so complex that one single biomarker is unlikely to capture the individual predisposition to disease development. The utility of adding multiple biomarkers from different disease pathways to predict the risk of death from cardiovascular cause has been explored in many studies and it is proving to be the only way to move forward (120–122). In their study, Halim *et al* associated a model combining 6 biomarkers (ICAM-1, MMP-3, NT-proBNP, IL-6, sCD40L, and IGFBP2) and 5 clinical variables (age, red-cell distribution width, diabetes, hemoglobin, and New York Heart Association class) with long term risk of cardiovascular events in terms of death and myocardial infarction (123).

However, the generation of multimarker panels must take several factors into consideration. For example, comparisons of biomarkers measured on the same set of individuals must account for their inherent correlation (people with high values of one marker will likely have high values of another) (124).

1.4.4 Defining abnormal biomarker values

Before assessing the clinical use of a biomarker, it is important to define abnormal values (116). Therefore, the distribution of the markers in people from a certain community needs to be characterised and especially in patients who will eventually benefit from the use of the tested biomarkers. Variations in biomarker levels with sex, age, ethnicity, co-morbidities and the disease itself must also be taken into account (125). There are different ways to define an abnormal biomarker level which are represented in Figure 5.

1- Reference limits: They are generally set with the use of cross-sectional analyses of a reference sample (usually a healthy sample free of the disease of interest), and an arbitrary percentile cutpoint (typically the 95^{th} or 97.5^{th} percentile) is chosen to define abnormality (126–128). Troponins cutpoint is 99^{th} percentile (52).

2-Discrimination limits: They are also used to indicate abnormal biomarker values. Such limits are generated by evaluating the degree of overlap between patients with and without disease in cross-sectional studies (129). As an example, when looking at the natriuretic peptides (NP) in a non-acute setting, a BNP plasma value >35 pg/mL and a NT-proBNP value of >125 pg/ml have been suggested as a threshold indicating heart failure and >125 pg/ml (130).

3-Threshold defining risk: A risk threshold identifies the level at which the risk of disease increases on follow-up. This method defines “undesirable” biomarker levels by associating values to the incidence of disease and seeking a threshold beyond which risk escalates (eg, systolic blood pressure ≤140 mm Hg and total cholesterol levels ≤4.9 mmol/L).

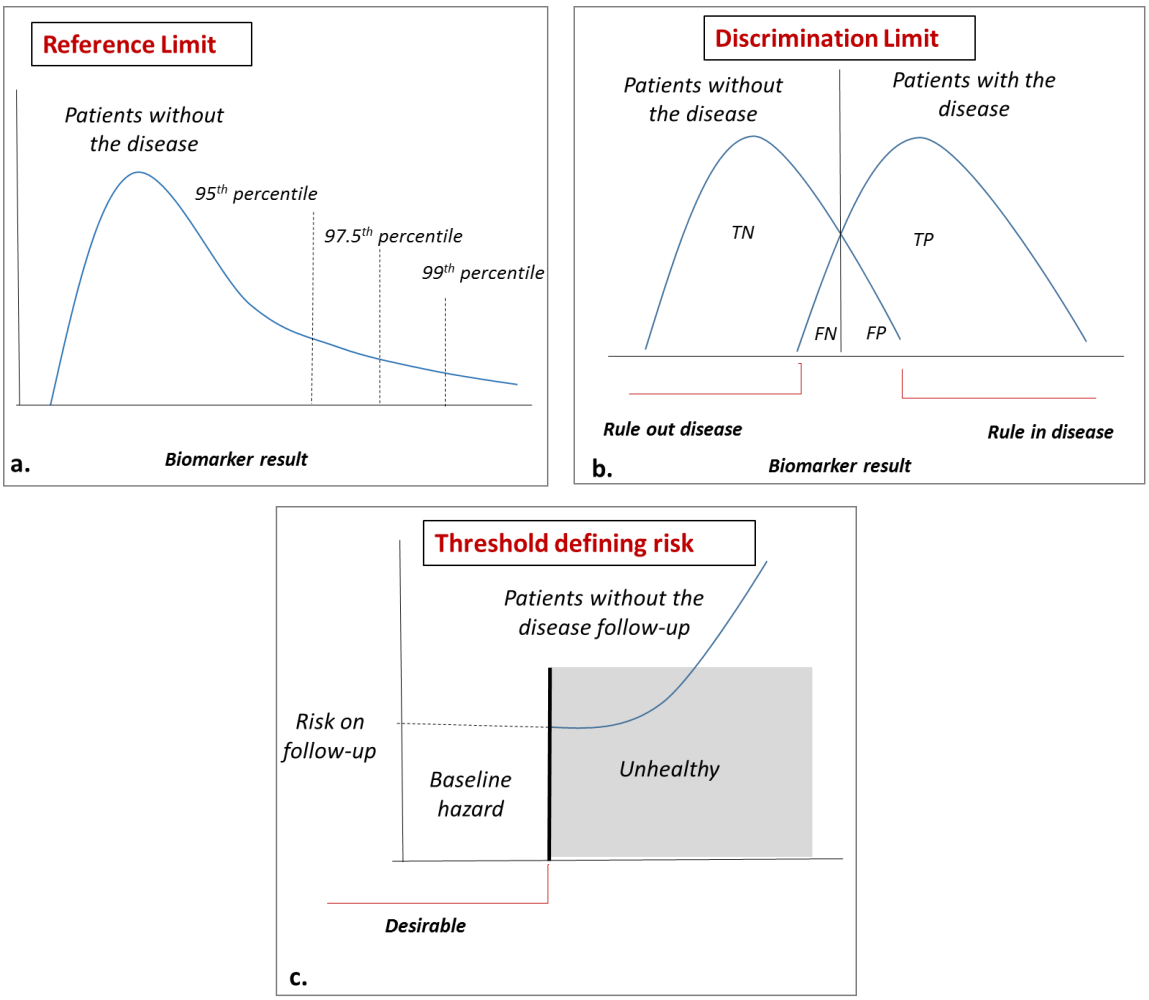


***Figure 5:* Three methods used to define abnormal values in biomarker development** *(Adapted from Vasan et al; 2006). TN: True negative; TP: True positive; FN: False negative; FP: False positive*

1.4.5 Laboratory factors in biomarker evaluation

In practice, biomarker performance needs to be assessed by analysing preanalytical, analytical, and postanalytical factors.

1.4.5.1 Preanalytical variability

Preanalytical variability refers to biomarker biological variability and stability over time (131,132). Preanalytical variability can be:

- Sample related: Type of sample collected, sample processing (anticoagulant added, platelet activation, temperature, degrading enzymes, freeze-thaw cycles), sample storage, half-life of the analyte.
- Assay related: Type of antibody used for the capture of the analyte, minimal detection limit, non-specific binding, false positive/false negative samples.
- Biological: Intra-individual (time of the sample, circadian rhythm, fasting state) or inter-individual (age, gender, drugs, diet, co-morbidities).

1.4.5.2 Analytical variability

Analytical variability relates to the performance of the test in the laboratory. Low analytical variability is a fundamental requirement of all biomarkers (133–135) , (Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS). Harmonized terminology database: 2005). Inter-laboratory variability (reagents used, calibration and analytical platforms) and intra-laboratory variability (staff-related) are important to consider.

Analytical variability is a combination of good accuracy and precision. In practice, if analytical imprecision is greater than biological variability, samples should be assayed in replicate, and quality control procedures that improve assay methodology and/or operating procedures should be implemented. This is critical for biomarkers used for point-of-care testing because imprecision may be greater in this setting compared with standard laboratory measurements. On the other hand, if biological variability is greater than analytical imprecision, the patient should be sampled on more than one occasion to obtain a true estimate of a biomarker. In order to reduce variability, it is important to limit multiple lots, replicate measurements, avoid repeated freeze thaw cycles, use a lab reference and a standard reference and regularly calibrate the instruments.

1.4.5.3 Postanalytical variability

Postanalytical factors that may affect biomarker performance, they include the processes of approval and transmission as well as the appropriate display of test results with the use of the laboratory's information management systems and the use of the test (automated platform in a centralised laboratory or point of care testing) (136).

1.4.6 Biomarker discovery

Biomarker discovery in CVD is very challenging because the patient vulnerable to CVD is most likely subject to several abnormalities: vulnerable plaque, vulnerable blood and vulnerable myocardium. In terms of developing biomarkers, two of these three components (vulnerable plaque and myocardium) are less directly accessible relative to the third (vulnerable blood). It is noteworthy to mention that in the case of atherosclerotic cerebrovascular disease, biomarkers may not be detectable in the peripheral circulation after a brain injury due to the presence of the blood-brain barrier. Thus, identifying the onset of stroke is quite challenging using diagnostic biomarkers (137).

There are two main strategies that are considered in CVD biomarker development which are complementary rather than mutually exclusive.

- **Deductive method or knowledge-based strategy** relies on exploring the biological processes involved in atherosclerosis and its evolution. It could consist of designing new assays for potential new biomarkers that are proven to be part of the atherosclerosis process or improving already existing biomarkers to improve their performance

- **Inductive strategy or unbiased strategy** screens through a large number of molecules in order to characterise the ones that are more likely to be linked to the disease profile.

Moreover, it is important to keep in mind that not every disease mediator is to be considered a biomarker. A particular analyte may participate clearly in a pathogenic pathway but not serve as an effective biomarker. As an example, soluble VCAM-1 does not predict the risk of future myocardial infarction in apparently healthy men (138). However, research has repeatedly and unequivocally demonstrated the essential role of VCAM-1 in experimental atherosclerotic lesion initiation and progression (139–143). Figure 6 details the five phases of biomarker development.

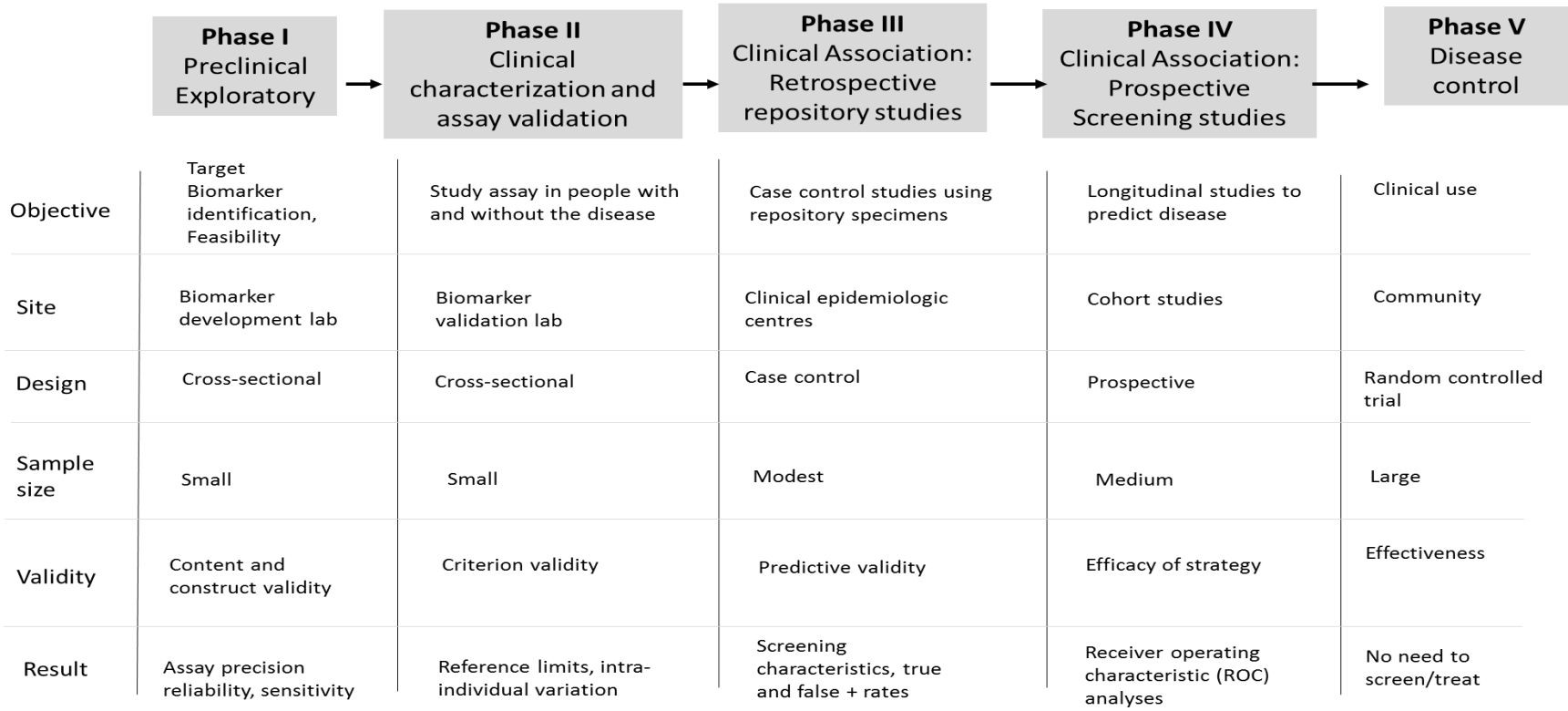


Figure 6*: *Five phases of biomarker development. *Adapted from Pepe et al, 2001* (144). *Content validity refers to the degree to which the biomarker represents the biological phenomenon studied (eg, serum CRP represents systemic inflammation); construct validity refers to establishing that the biomarker is measuring the aspect of disease (some conceptual construct or theory) that we want to measure (eg, we want to measure plaque inflammation; therefore, we should establish whether serum CRP relates to atherogenesis and plaque inflammation); and criterion validity refers to the how well the biomarker identifies disease state when compared with a gold standard (measured in terms of sensitivity and specificity; eg. how well does CRP predict CVD?).*

1.5 The need for new risk prediction tools: Exploring the TACE/TNFα pathway and new biomarkers using a proteomic approach

Biomarkers are powerful tools that enable the understanding of the spectrum of CVD with applications in at least five areas: screening, diagnosis, prognostics, prediction of disease recurrence, and therapeutic monitoring. Although advances in functional genomics, proteomics, metabolomics, and bioinformatics have revolutionised the new era of personalised medicine, the translation of biomarker research into clinical practice is proving to be very difficult.

The already available CVD biomarkers present many issues. High sensitivity C reactive protein lacks specificity and is known to be elevated in several inflammatory conditions (145). Troponins, on the other hand, give an idea of the extent of the myocardial damage, however, they are only elevated when the myocardial necrosis has already taken place and can also be elevated in other conditions highlighted in Table 2 (146). Some characteristics revealed by the ECG hold a prognostic value, however, these changes are a result of an ACS and are ineffective when it comes to primary prevention (147). At present, there is no efficient blood test that allows the clinicians to know which patients are at higher risk of acute events before they have had their first or second event. In an ideal world, a coronary angiogram would be performed on everyone which wouldn't be cost-effective. In addition, coronary angiograms are unable to detect any newly formed lesions that haven't developed yet into a proper plaque (148).

CVD risk factors such as family history, diabetes, blood pressure, chronic kidney disease, hypercholesterolemia, smoking and diet are among the characteristics that clinicians rely on to treat patients at higher risk of developing CVD and are normally

combined into risk scores. However, many people who are mistakenly considered at low risk eventually develop an ACS (149) and it was also shown that traditional risk factors only explain 50% of CVD risk (64,150). Recently, the concept of one ideal biomarker is becoming rather faint and the idea of multi-marker profiling is emerging. The development of a biomarker panel that explores a multitude of inflammatory pathways know to be involved in different stages of CVD initiation, progression and complications is the only way to improve current risk prediction models.

The inflammatory pathway involving Tumour necrosis factor alpha converting enzyme (TACE) and tumour necrosis factor alpha (TNFα) has been previously investigated in CVD. As many studies have shown, TACE is responsible for the ectodomain shedding of a variety of inflammatory markers, including TNFα, most of which play role in the initiation and progression of CVD (151–156). The number of known TACE substrates continues to increase with mounting evidence that TACE is implicated in many cellular functions. Recent research indicates a particular role for TACE and the TNF family members in CAD and MACE, but none have really looked at this panel from a biomarker development point of view. Further investigations are however required to ascertain the exact role and mechanism of action of TACE in this disease area. Future prospective studies in clinical cohorts at varying degrees of MACE risk stratification are needed to fully assess TACE as a potential biomarker for CAD and MACE risk. This will be crucial for the future development of new personalised predictive tests and therapeutics that can improve patient clinical care pathways and prevent the high mortality rates associated with CAD (Figure 7). The rationale for investigating TACE inflammatory pathway is detailed in the literature review that is included **in section IV of the appendix** (157).

On the other hand, recent advances in proteomics could enable the transition from the single marker to the multimarker approach. This will allow the identification of markers of CVD risk as well as markers to further stratify patients with established CVD (158).

1.6 Hypothesis

By exploring a large number of proteins from several inflammatory pathways involved in CVD in individuals at varying levels of CVD risk, it is hypothesised that it will be possible to discover multimarker panels that can identify individuals at risk of cardiovascular events and further stratify those people at very high risk of major adverse cardiovascular events. Specifically, this project hypothesises that by adding an increasing number of promising biomarkers to such a multimarker panel will improve the current CVD risk assessment tools. To test this hypothesis, first, the well-known inflammatory pathway involving tumour necrosis factor alpha converting enzyme (TACE) and tumour necrosis factor alpha (TNFα) is explored. Secondly, a proteomic approach is undertaken to uncover other known and novel markers of cardiovascular and MACE risk.

1.7 Aims

The aims of this present work are to:

1.7.1 Evaluate the value of proteins from TNFα inflammatory pathway in cardiovascular disease (CVD) risk assessment and stratification

By exploring a number of proteins that are part of the TNFα inflammatory pathway, it could be possible to characterise participants at very high risk of CVD.

1.7.2 Investigate the value of a panel combining 10 inflammatory markers in CVD risk assessment

Measuring circulating levels of 10 inflammatory proteins in participants at various levels of CVD risk could improve current CVD risk assessment.

1.7.3 Determine whether a proteomic approach combining 184 proteins could improve the current CVD risk assessment scores

Exploring a large number of inflammatory proteins could highlight underlying biological and pathological CVD pathways that might be useful to include in multimarker panels for CVD risk assessment or might prove to be potential therapeutic targets.

1.7.4 Explore whether potential proteins are useful in further stratifying very high risk CVD participants in terms of recurrent major adverse cardiovascular events (MACE)

Since very high risk CVD individuals are at high risk of MACE, it could be useful to identify which proteins are associated with MACE risk over time

1.7.5 Assess whether candidate proteins for biomarker development are subject to experimental and technical variability

In order to assess the value of a protein as a potential biomarker, levels could be evaluated in different experimental and technical conditions as a validation step before their validation in larger cohorts.

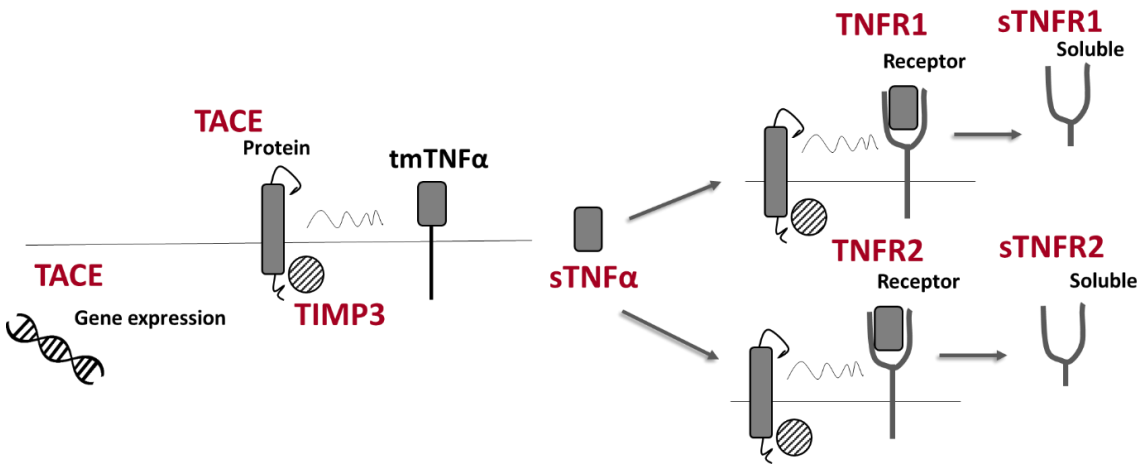


***Figure 7*: TACE/TNFα pathway.** *TACE, once synthesised cleaves transmembrane TNFα (tmTNFα) releasing soluble TNFα (sTNFα). TACE is inhibited by Tissue Inhibitor of Metalloproteinase 3 (TIMP3). sTNFα binds to TNF receptor 1 (TNFR1) and 2 (TNFR2). TACE also cleaves TNFR1 and TNFR2 releasing their respective soluble forms, soluble TNFR1 (sTNFR1) and soluble TNFR2 (sTNFR2). TACE: tumour necrosis factor alpha converting enzyme; TIMP3: Tissue Inhibitor of Metalloproteinase 3; tmTNFα: transmembrane tumour necrosis factor alfa; sTNFα: soluble tumour necrosis factor alfa; TNFR1: tumour necrosis factor alfa receptor 1; TNFR2: tumour necrosis factor alfa receptor; sTNFR1: soluble tumour necrosis factor alfa receptor 1; sTNFR2: soluble tumour necrosis factor alfa receptor 2; TNFα: tumour necrosis factor alfa.*

Chapter 2

Patients, Materials and Methods

2.1 Methods for participant recruitment

The study was approved by the Office for Research Ethics Committees Northern Ireland (ORECNI). The ethics research reference number was 14/NI/0068. The study was conducted in accordance with the declaration of Helsinki and patients were recruited from November 2014 until March 2016.

2.1.1 Participants recruited from Altnagelvin Area Hospital

Participants were recruited consecutively from the catheterisation laboratory in Altnagelvin Area Hospital (cardiology unit (CCU) and day care unit (CDU)). Participants were consented prior to the blood sample collection and given the participant information sheet. A short questionnaire was completed which included the patient's medical history, family history and lifestyle questions such as smoking, diet and exercise (Figure 1 and 2). Blood pressure was recorded at the time of consent. C reactive protein (CRP) and electrolytes levels were also measured on the day. Any missing information (such as medication history, blood test results and procedure outcome) was completed at a later stage by accessing the Northern Ireland Electronic Care Record (NIECR) with the help of cardiologists and cardiac research nurse (Figure 2). Recruited participants were identified as either acute coronary syndrome (ACS) or elective participants (ELEC) (Figure 1). ACS participants attended hospital either on the day or in the previous couple of days and were admitted for unstable angina (UA), non-ST segment elevation myocardial infarction (NSTEMI) or ST segment elevation myocardial infarction (STEMI) (Figure 1). ELEC participants attended the hospital for a scheduled cardiac angiogram to investigate a potential underlying coronary artery disease (CAD) behind their angina symptoms. These participants were then classified according their angiogram result and

their medical history using the SCORE risk chart as per the European Society of Cardiology (ESC) guidelines. Refer to **section 2.1.3.**

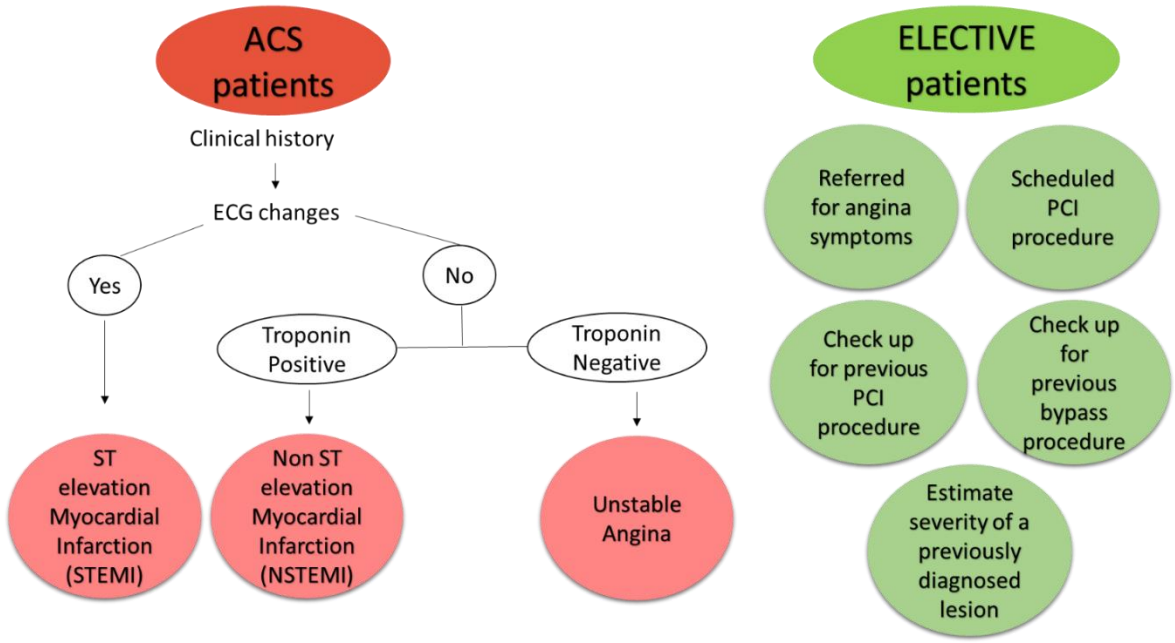


***Figure 1:* Participants with an Acute Coronary Syndrome (ACS) or admitted electively (ELEC) that took part in the study**. *ACS: Acute coronary syndrome; ECG: Electrocardiogram; PCI: percutaneous coronary intervention.*

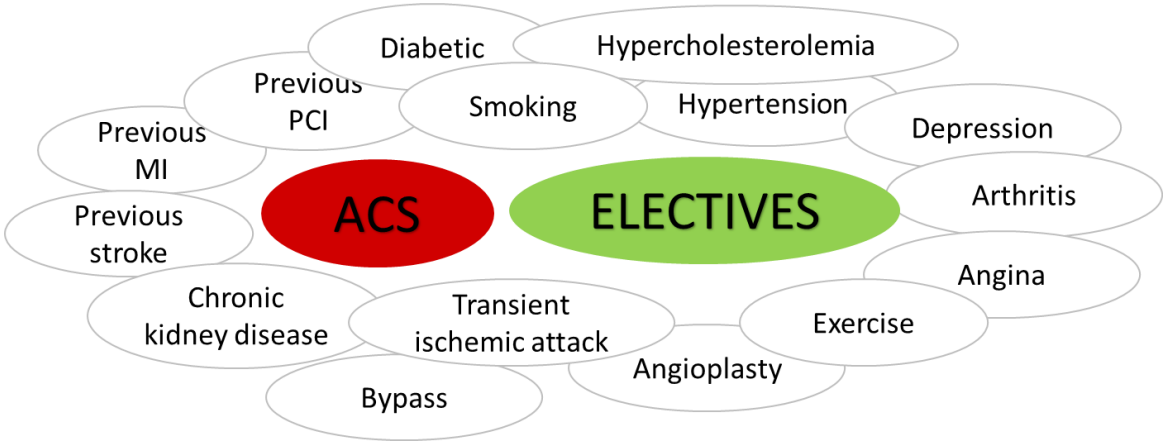


***Figure 2:* Participants co-morbidities recorded in the questionnaire.** *ACS: Acute coronary syndrome; MI: myocardial infarction; PCI: percutaneous coronary intervention.*

2.1.2 Participants recruited among the Western Trust and Ulster University staff

Participants were recruited *via* an email advertisement to the Western Trust and Ulster University staff across Northern Ireland. Interested participants were provided with participant information sheet and an appointment was arranged. After giving informed consent, a blood sample was collected followed by completion of the lifestyle questionnaire. Blood pressure was also recorded and CRP levels, total cholesterol and a full blood count were measured on the day. These participants were then stratified according to the ESC guidelines and a SCORE was assigned.

2.1.3 Methods for scoring participants according to the SCORE risk chart (ESC guidelines)

The European low risk Systematic COronary Risk Evaluation (SCORE) risk score chart was used to determine the 10-year risk of fatal CVD in low risk regions of Europe by gender, age, systolic blood pressure, total cholesterol and smoking status for all recruited individuals who were from Ireland. To include the effect of HDL cholesterol levels when calculating the SCORE risk, the online HeartScore system was used (heartscorre.org). Group1 were defined as very high risk participants (VHR) with a 10-year risk SCORE ≥10% risk of fatal CVD and had evidence of established CAD as per coronary angiography. VHR participants were subdivided in Acute Coronary Syndrome participants (ACS-VHR) and elective percutaneous coronary intervention participants (ELEC-VHR). Group 2 were identified as low, moderate and high risk participants referred to collectively as non-VHR with a 10-year risk SCORE <10% risk of fatal CVD. Within the non-VHR category, low risk (LR) participants had a 10-year risk SCORE of <1% of fatal CVD, moderate risk (MR)

participants had a 10-year risk SCORE of ≥ 1% and <5% of fatal CVD and high risk (HR) participants had a SCORE of ≥5% and <10% of fatal CVD.

The European low risk SCORE chart was used to determine the 10-year risk of fatal cardiovascular disease (CVD) in low risk regions of Europe by gender, age, systolic blood pressure, total cholesterol and smoking status for all recruited individuals who were from the UK and Ireland. To estimate a person's 10-year risk of CVD death, the table corresponding to the individual's gender, smoking status, and age was selected. Within this table the cell nearest to the individual's blood pressure and total cholesterol was selected. In order to include the effect of HDL cholesterol and the effect of age when calculating the SCORE risk, the online HeartScore system was used (heartscorre.org).

Participants with the following criteria were automatically considered at very high risk (VHR) (as per the ESC guidelines) (63) (Figure 3):

- Documented CVD by invasive or non-invasive testing (such as coronary angiography, nuclear imaging, stress echocardiography, carotid plaque on ultrasound), previous myocardial infarction (MI), ACS, coronary revascularization [percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG)] and other arterial revascularization procedures, ischaemic stroke, peripheral artery disease (PAD).
- Type 2 diabetes, participants with type 1 diabetes with target organ damage (such as microalbuminuria).
- Moderate to severe chronic kidney disease [glomerular filtration rate (GFR) < 60 mL/min/1.73 m2).
- A calculated 10-year risk SCORE ≥10%

High risk (HR) subjects were characterised with any of the following:

- Markedly elevated single risk factors such as familial dyslipidaemias and severe hypertension.
- A calculated SCORE ≥5% and <10% for 10-year risk of fatal CVD.

Moderate risk (MR) subjects were considered to be at moderate risk when their SCORE was ≥1% and <5% at 10 years.

The low risk (LR) category applied to individuals with SCORE < 1%.

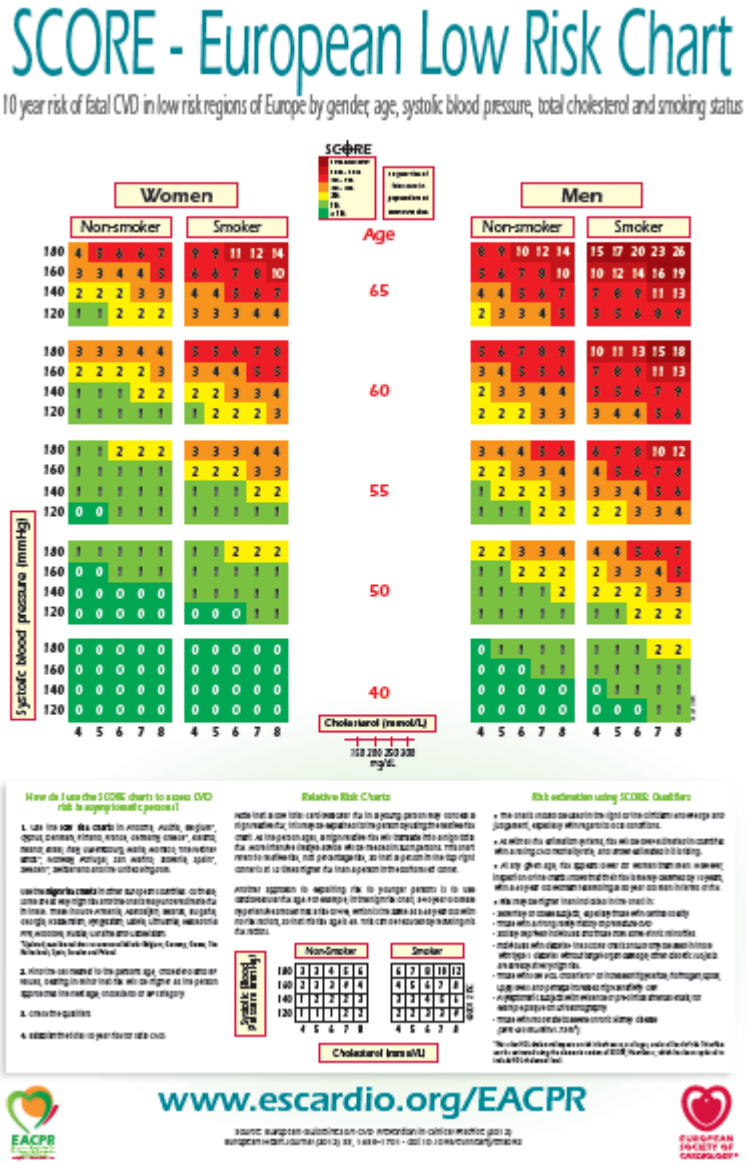


***Figure 3:* EUROSCORE low risk chart**

2.1.4 Methods for recruiting follow up participants

A patient information sheet was posted to those that were already recruited and consented to a willingness to take part in future studies. These participants where contacted by phone after two weeks to assess their interest in volunteering to provide a follow up sample for the study. An appointment was scheduled for the interested participants. During this appointment, a blood sample was collected and the blood pressure was recorded.

2.2 Methods for blood sample collection

A total volume of 35 ml of blood was collected from all recruited participants (apart from the sub studies and the follow up participants). This volume was divided as follows (Figure 4):

- 3 x 8 ml of EDTA tubes for the research lab – VACUETTE K3E K3EDTA – 455036
- 1 x 4 ml of serum tube for the research lab – VACUETTE Z serum clot activator - 454092
- 1 x 4 ml of EDTA tube for hospital lab (to measure the full blood count (FBC)) – VACUETTE K3E K3 EDTA – 454036
- 1 x 3.5 ml of serum separator tube (yellow cap) (to measure the cholesterol) – BD Vacutainer SST^{TM} II advance - 367956

For the participants recruited from the catheterisation lab, blood was collected from the sheath inserted into the radial artery (or, on occasions, the femoral artery when the radial artery couldn't be accessed). The arterial blood was collected straight into a 50-ml syringe and immediately transferred into blood tubes using a 21 G syringe (BD microlane 3 – 21G 1 ½ Nr.2 0.8 x 40 mm – 304432).

Participants recruited among the Western Trust and Ulster University staff were received at the clinical translational research and innovation centre (C-TRIC). A 35 ml of venous blood was collected by a trained phlebotomist using a butterfly needle (VACUETTE safety blood collection set 21G x 3/4 '' 0.9 x 19 mm – 450091) as well as a luer adapter (Greiner bio one luer adapter – 20G 16C05A) and a BD Vacutainer.

For a subset of participants recruited from the catheterisation lab (n=39), venous blood and arterial blood was collected (a total of 40 ml was collected). This was to compare the effect of the sampling site on the studied markers. The venous blood was collected by a trained phlebotomist prior to the angiogram procedure using the same material mentioned above.

For another subset of participants recruited from the catheterisation lab (n=6), blood was collected from different sampling sites by the consultant cardiologist (10 ml from each site):

- Peripheral artery (PA) (radial or femoral artery)
- Central vein (CV) (femoral vein)
- Central coronary artery (CCA)
- Culprit lesion (when present)
- Coronary sinus
- Post-balloon sample
- Post-PCI sample
- Post-heparin sample

From the follow up participants (n=16), 8 ml of venous blood was collected by a trained phlebotomist using the same material mentioned above.

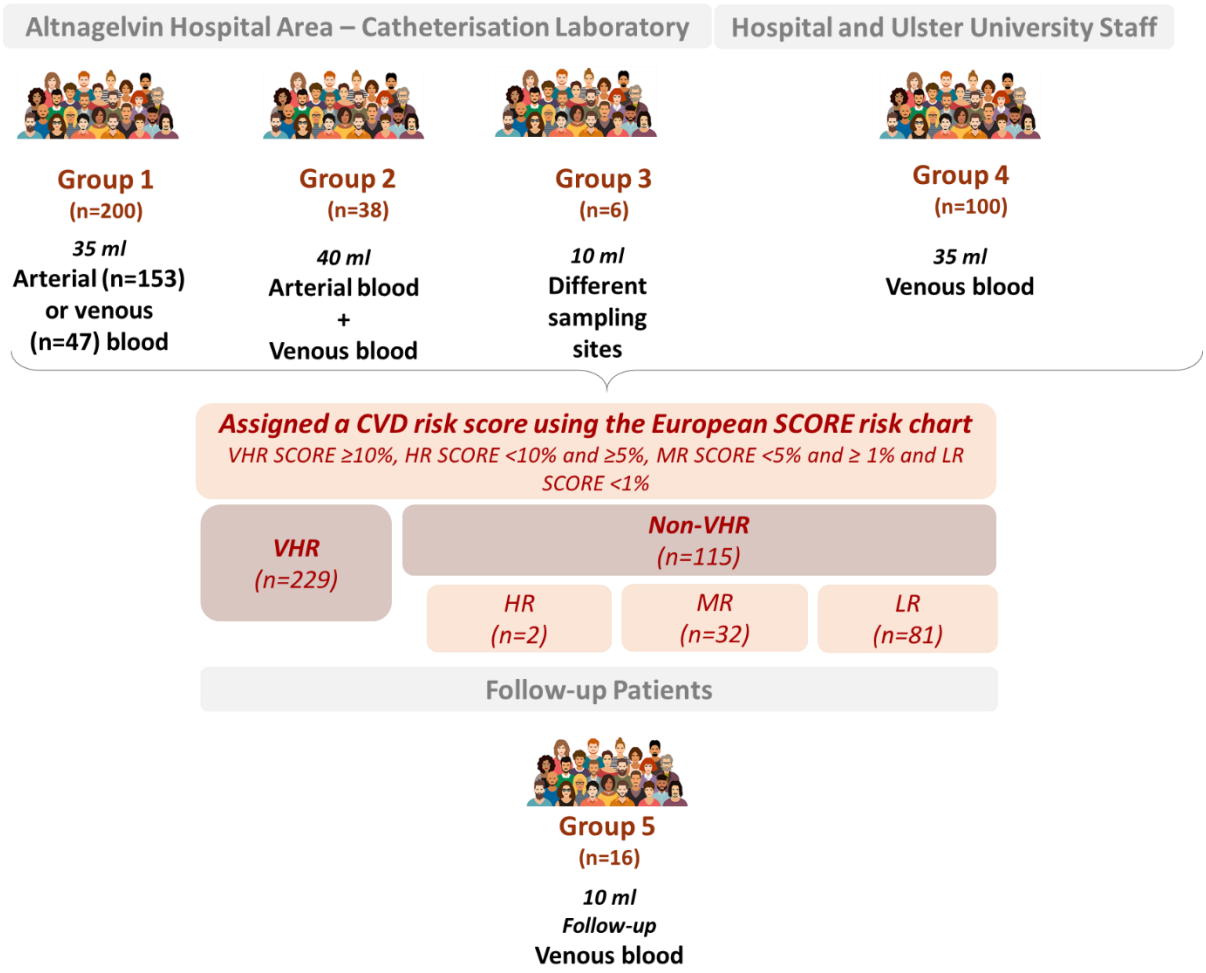


***Figure 4*: Types and volumes of the blood samples collected from patients recruited from the catheterisation laboratory in Altnagelvin Area Hospital and from the hospital and Ulster University staff.** *CVD: Cardiovascular disease, HR: high risk, LR: low risk, MR: moderate risk, SCORE: Systematic COronary Risk Evaluation; risk, VHR: very high*

A saliva sample was also collected when possible from recruited participants, although not used in the current study but as part of a larger study. The saliva sample was taken using the Omnigene oral kit for collection of microbial RNA and DNA OM-505. The sample was left a room temperature for no longer than 3 weeks before aliquoting and freezing the aliquots at -80◦C.

2.3 Methods for spinning, aliquoting and storing blood samples

After the blood samples were collected, the blood tubes were kept on ice (or at 4◦C) until processing. The blood was processed within 4 hours of collection. Plasma (from 2 EDTA 8 ml tubes) and serum (4 ml) tubes were spun down at 2000 relative centrifugal force (RCF), at 4◦C for 15 minutes. Plasma and serum were then aliquoted into 500 ul aliquots and stored at -80◦C for further downstream applications.

Buffy coat from both EDTA tubes (8 ml each) were collected and washed twice with 1X phosphate buffer saline (PBS). Once washed, one buffy coat was then resuspended 1 ml of RNAlater® Stabilization Solution (AM7020). RNAlater® RNA Stabilization Solution stabilises and protects cellular RNA in intact, unfrozen tissue samples, eliminating the need to immediately process tissue samples or to freeze samples in liquid nitrogen for later processing. Samples were then stored at -80◦C for further RNA extraction. The other washed buffy coat was suspended in 1 ml of M-PER™ Mammalian Protein Extraction Reagent (78501) and immediately placed on ice and then stored at -80◦C until further protein extraction. M-PER Mammalian Protein Extraction Reagent is a mild detergent lysis that extracts soluble proteins in non-denatured state. The remaining 8 ml EDTA tube was stored as it is at -80◦C until further DNA extraction (whole blood tube). Refer to Figure 5 for a diagram summary of blood processing.

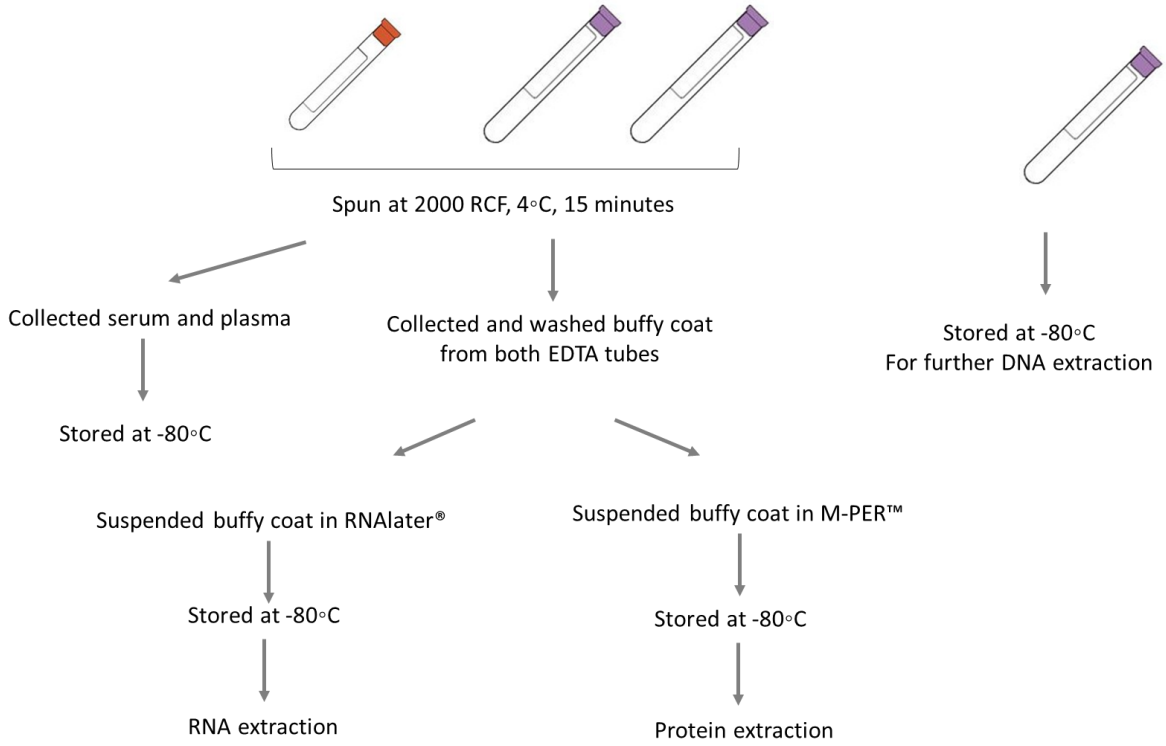


Figure 5: ***Processing of the blood tubes until further analysis.*** *EDTA: Ethylenediaminetetraacetic acid; M-PER: Mammalian Protein Extraction Reagent; RCF: relative centrifugal force*

Sample aliquots were all stored into Sarstedt® boxes and were divided into a primary and a backup -80 alarmed freezers. A sample map was created for all the samples to track the sample location, the number of thaws (samples were thawed no more than 3 times), the volume used, the volume left and the type of experiment that was performed on each aliquot. All samples were handled according to the Good Clinical Practice (GCP) and the Human Tissue Act (HTA) recommendations.

<u>Table 1:</u> TACE, TNFR1, TNFR2 and TIMP3 protein levels measured in different types of samples

Type of sample	TACE	sTNFR1	sTNFR2	TIMP3
Arterial	✓	✓	✓	✓
Venous	✓	✓	✓	✓
Arterial and venous	✓	✓	✓	✓
Different sampling sites	✓	✓	✓	✓
Serum	✓			
Timepoint 1	✓	✓	✓	✓
Timepoint 2	✓	✓	✓	✓
Spun down plasma	✓			
Fresh plasma	✓			

TACE protein levels were measured in arterial samples, venous samples, arterial and venous samples from the same patients, in different sampling sites, in the serum, on timepoint one and 2, in plasma samples after being spun down, and in plasma samples collected on the day. TNFR1, TNFR2 and TIMP3 protein levels were measured in arterial samples, venous samples, arterial and venous samples from the same patients, in different sampling sites, on timepoint one and 2. TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: Metalloproteinase inhibitor 3; TNFα: Tumour necrosis factor alpha; TNFR1: Tumour necrosis factor alpha receptor 1; TNFR2: Tumour necrosis factor alpha receptor 2.

2.4 Method for extracting proteins stored in M-PER™ Mammalian Protein Extraction Reagent (#78501) – ThermoFisher Scientific, (Illinois, USA) - in -80◦C

This procedure was followed according to the manufacturer's instructions. Frozen samples in M-PER® were slowly thawed on ice. The mixture was then shaken gently for 10 minutes and the cell debris was removed by centrifugation at 15,000 RCF for 15 minutes. The supernatant was then transferred to a new tube and immediately stored at -80◦C.

Note: M-PER® Reagent does not contain protease inhibitors. The effect of adding a protease inhibitor before freezing or after thawing the samples did not significantly

change the amount of proteins recovered and did not affect downstream applications such as the BCA assay used for protein quantification.

***Table 2*: Different methods for proteins extractions and protein yield.**

Type of sample (MD013)	Protein concentrations (BCA) (ug/ml)
Proteins extracted after freezing down buffy coat	
Buffy coat + M-PER at -80◦C	14637.284
Buffy coat + Protease inh. + MPER at -80◦C	16312.372
Buffy coat + M-PER at -80◦ add protease inh. after thawing (before analysis)	9594.396
Proteins extracted from fresh buffy coat	
Fresh buffy coat + M-PER and extract proteins	15009.334
Fresh buffy coat + M-PER + protease inh. and extract proteins	5217.474

BCA: bicinchoninic acid assay; M-PER: Mammalian Protein Extraction Reagent; Protease inh.: protease inhibitor

2.5 Method for quantifying proteins using the bicinchoninic acid (BCA) assay protein assay kit (23225) – Pierce Thermo Scientific, (Illinois, USA)

BCA was performed according to the manufacturer's instructions in a 96-well plate. Standards were prepared by diluting the contents of one Albumin Standard (BSA) ampoule into several clean vials and adding the appropriate volume of diluent (PBS was used in this case). The volume of working reagent needed was determined per the number of samples to quantify using the following formula:

(number of standards + number of samples) × (number of replicates) × (200 ul of working reagent) = total volume working reagent (WR) required

The working reagent (WR) was then prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). In order to reduce the volume of sample

used, 20 ul of PBS was mixed with 5 ul of standard or sample and then 200 ul or working reagent was added to each well. The plate was placed on a plate shaker for 30 seconds and then incubated at 37◦C for 30 minutes. The plate was then read at 562 nm.

For creating the standard curve and calculating the sample concentrations, the following software was used: https://www.myassays.com/

Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again. A new standard curve was made for each plate.

Note: The coefficient variation (CV) is the ratio of the standard deviation to the mean: CV = st.dev/mean

Note: Samples with a protein concentration out of the range of the standard curve required a 10-fold dilution. The calculated concentration was then multiplied by 10 in order to get the final concentration.

2.6 TACE ELISA

2.6.1 Method for measuring tumour factor necrosis alpha converting enzyme (TACE) protein levels in the plasma by ELISA - Human TACE ELISA Kit - RAB0003 SIGMA, (Missouri, USA)

TACE ELISA was performed according to the manufacturer's instructions. Samples were diluted 2-fold in assay/sample diluent buffer (buffer C - item L - RABELADC-30ML); (200 ul of sample + 200 ul of diluent). The 8 standards were prepared by serial dilution in the diluent with 5000 pg/ml as the highest standard and only using the assay/sample diluent

buffer for the lowest standard concentration (5000 pg/ml; 2500 pg/ml; 1250 pg/ml; 625 pg/ml; 312.5 pg/ml; 156.3 pg/ml; 78.15 pg/ml; 0 pg/ml). All reagents were at room temperature before starting the experiment (which was between 22◦ and 28◦C).

Samples were run in triplicate and 100 ul of diluted sample or prepared standard were added per well. Wells were covered and incubated for 2.5 hours at room temperature (between 22◦C and 28◦C) with gentle shaking. The solution was discarded and the plate was washed 4 times using the 1X prepared wash buffer (initially at 20X – diluted with deionised water) and blotted against paper towels to remove any remaining wash buffer. A volume of 100 ul of 1X prepared biotinylated detection antibody (diluted 80-fold with prepared diluent buffer B - Item E1 - RABELADB-15ML) was added to each well and the plate was incubated for one hour at room temperature with gentle shaking.

The solution was discarded and the plate was washed 4 times using the 1X prepared wash buffer and blotted against paper towels to remove any remaining wash buffer. A volume of 100 ul of 1X prepared HRP-Streptavidin solution (diluted 200-fold with prepared diluent buffer B –Item E1 - RABELADB-15ML) was added to each well and the plate was incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and the plate was washed 4 times using the 1X prepared wash buffer and blotted against paper towels to remove any remaining wash buffer. A volume of 100 ul of ELISA Colorimetric TMB Reagent (Item H - RABTMB3) was added to each well and the plate was Incubated for 30 minutes at room temperature in the dark with gentle shaking. A volume of 50 ul of Stop Solution (Item I - RABSTOP3) was added to each well and the plate was read immediately at 450 nm.

For the result analysis, the mean absorbance was calculated for each set of triplicate standards and samples. A standard curve was created from the standards using a 4-

parametric logistic regression curve. The sample concentration was then calculated after multiplying by the dilution factor. For creating the standard curve and calculating the sample concentrations, the following website was used: https://www.myassays.com/. Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again. A new standard curve was made for each plate.

Note: When detecting low concentrations between 200 and 0 pg/ml, the CVs were higher than usual. The sensitivity of the TACE ELISA kit provided by the manufacturer was 50 pg/ml.

TACE ELISA inter-assay CV = 19.68 %

To calculate the inter assay CV (%), a sample was measured on two occasions on different plates and an average mean was calculated on each of those occasions. The standard deviation (SD) was calculated from those means followed by the CV (SD divided by the average of both measurements). The intra-assay CV was then calculated by averaging the individually calculated CVs.

<u>Table 3:</u> Inter-assay coefficient of variation (CV) of TACE ELISA for the measurement of TACE plasma protein levels

Study ID	Measurment 1 (mean in pg/ml)	Day after measurment 1	Measurment 2 (mean in pg/ml)	Day after measurment 2	Average	Stdev of means	CV	CV (%)
VHR-C-003	8571	208	2121	468	5346	4560.839	0.853131	85.31311
VHR-C-004	9656	205	7361	468	8508.5	1622.81	0.190728	19.07281
VHR-C-057	1607	281	1198	15	1402.5	289.2067	0.206208	20.6208
VHR-C-067	313.4	21	541.4	531	427.4	161.2203	0.377212	37.72119
VHR-C-068	473.9	20	433	530	453.45	28.92067	0.063779	6.377918
VHR-C-069	2398	20	2604	530	2501	145.664	0.058242	5.82423
VHR-C-070	3389	20	3763	530	3576	264.4579	0.073954	7.395356
VHR-C-071	1568	17	1922	527	1745	250.3158	0.143447	14.34475
VHR-C-121	2992	24	2881	155	2936.5	78.48885	0.026729	2.672871
VHR-C-143	475	15	493.4	128	484.2	13.01076	0.026871	2.687064
VHR-C-150	1575	36	1027	373	1301	387.4945	0.297844	29.78436
VHR-C-183	512.8	10	644	78	578.4	92.77241	0.160395	16.03949
VHR-C-184	1796	10	1807	78	1801.5	7.778175	0.004318	0.431761
VHR-C-185	131.9	10	234.4	78	183.15	72.47845	0.395733	39.57327
VHR-C-186	1847	10	2555	78	2201	500.6316	0.227456	22.74564
MD-001	3140		3342		3241	142.8356	0.044071	4.407145
Inter-assay CV (%) (n=15)								**19.68823**

TACE ELISA intra-assay CV = 11.91 %

To calculate the intra assay CV (%), samples were analysed in triplicates, the mean concentration was calculated for each sample, standard deviation (SD) was calculated. The CV was then calculated by dividing the SD by the mean and then converted into percentage. The inter-assay CV was calculated by averaging the individually calculated CVs. (Missing CVs refer to values that were below the limit of detection of the assay).

<u>Table 4:</u> Intra-assay coefficient of variation (CV) of TACE ELISA for the measurement of TACE plasma protein levels

Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)
VHR-C-001	8.6	VHR-C-026	-	VHR-C-051	-	VHR-C-076	5.6	VHR-C-101	-	VHR-C-126	-	VHR-C-151	-
VHR-C-002	-	VHR-C-027	-	VHR-C-052	-	VHR-C-077	20.2	VHR-C-102	-	VHR-C-127	-	VHR-C-152	-
VHR-C-003	5.8	VHR-C-028	-	VHR-C-053	8.37	VHR-C-078	-	VHR-C-103	3.48	VHR-C-128	-	VHR-C-153	37.4
VHR-C-004	1.37	VHR-C-029	-	VHR-C-054	10.74887	VHR-C-079	22.70769	VHR-C-104	4.81	VHR-C-129	4.5	VHR-C-154	0.922
VHR-C-005	-	VHR-C-030	4.37	VHR-C-055	-	VHR-C-080	-	VHR-C-105	-	VHR-C-130	6.899093	VHR-C-155	4.34
VHR-C-006	-	VHR-C-031	5.22	VHR-C-056	3.452825	VHR-C-081	-	VHR-C-106	-	VHR-C-131	-	VHR-C-156	-
VHR-C-007	-	VHR-C-032	-	VHR-C-057	2.96	VHR-C-082	2.06	VHR-C-107	-	VHR-C-132	-	VHR-C-157	0.477
VHR-C-008	-	VHR-C-033	-	VHR-C-058	17.2	VHR-C-083	7.65	VHR-C-108	-	VHR-C-133	24.68264	VHR-C-158	-
VHR-C-009	-	VHR-C-034	-	VHR-C-059	2.79	VHR-C-084	-	VHR-C-109	-	VHR-C-134	-	VHR-C-159	-
VHR-C-010	11.8	VHR-C-035	2.01	VHR-C-060	-	VHR-C-085	-	VHR-C-110	45.8	VHR-C-135	1.99	VHR-C-160	-
VHR-C-011	24.8	VHR-C-036	-	VHR-C-061	-	VHR-C-086	-	VHR-C-111	12.1	VHR-C-136	9.35	VHR-C-161	29.8
VHR-C-012	-	VHR-C-037	-	VHR-C-062	-	VHR-C-087	-	VHR-C-112	-	VHR-C-137	-	VHR-C-162	21.28755
VHR-C-013	-	VHR-C-038	-	VHR-C-063	-	VHR-C-088	3.75	VHR-C-113	3.87	VHR-C-138	3.97	VHR-C-163	10.90123
VHR-C-014	-	VHR-C-039	-	VHR-C-064	36.2	VHR-C-089	-	VHR-C-114	23.2	VHR-C-139	-	VHR-C-164	-
VHR-C-015	-	VHR-C-040	-	VHR-C-065	-	VHR-C-090	-	VHR-C-115	13.4	VHR-C-140	-	VHR-C-165	-
VHR-C-016	10.9	VHR-C-041	2.75	VHR-C-066	-	VHR-C-091	-	VHR-C-116	11.15664	VHR-C-141	20	VHR-C-166	-
VHR-C-017	-	VHR-C-042	-	VHR-C-067	12.2	VHR-C-092	-	VHR-C-117	-	VHR-C-142	19.4	VHR-C-167	30
VHR-C-018	3.77	VHR-C-043	-	VHR-C-068	4.71	VHR-C-093	-	VHR-C-118	19.8	VHR-C-143	15.4	VHR-C-168	-
VHR-C-019	-	VHR-C-044	16.3	VHR-C-069	3.41	VHR-C-094	6.3	VHR-C-119	5.18	VHR-C-144	-	VHR-C-169	18.2
VHR-C-020	-	VHR-C-045	-	VHR-C-070	3.64	VHR-C-095	-	VHR-C-120	-	VHR-C-145	-	VHR-C-170	-
VHR-C-021	7.83	VHR-C-046	23.4	VHR-C-071	1.17	VHR-C-096	-	VHR-C-121	1.35	VHR-C-146	35.9	VHR-C-171	-
VHR-C-022	-	VHR-C-047	-	VHR-C-072	41.3	VHR-C-097	-	VHR-C-122	-	VHR-C-147	43.9	VHR-C-172	-
VHR-C-023	-	VHR-C-048	1.28	VHR-C-073	-	VHR-C-098	-	VHR-C-123	22.6	VHR-C-148	-	VHR-C-173	-
VHR-C-024	-	VHR-C-049	5.37	VHR-C-074	-	VHR-C-099	-	VHR-C-124	-	VHR-C-149	2.82	VHR-C-174	-
VHR-C-025	-	VHR-C-050	-	VHR-C-075	6.96	VHR-C-100	-	VHR-C-125	-	VHR-C-150	6.33	VHR-C-175	-
Patient ID	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**
VHR-C-176	-	VHR-C-201	7.71	LR-1	10.5	LR-26	-	LR-51	-	LR-76	30	No-CAD 1	-
VHR-C-177	24.6	VHR-C-202	-	LR-2	7.09	LR-27	6.37	LR-52	1.27	LR-77	6.79	No-CAD 2	-
VHR-C-178	-	VHR-C-203	-	LR-3	2.69	LR-28	-	LR-53	7.96	LR-78	5.27	No-CAD 3	-
VHR-C-179	-	VHR-C-204	17.7	LR-4	27.3	LR-29	1.51	LR-54	24.8	LR-79	-	No-CAD 4	2.86
VHR-C-180	22.7	VHR-C-205	3.07	LR-5	-	LR-30	-	LR-55	-	LR-80	9.67	No-CAD 5	5.02
VHR-C-181	-	VHR-C-206	-	LR-6	-	LR-31	-	LR-56	-	LR-81	6.54	No-CAD 6	-
VHR-C-182	4.411842	VHR-C-207	-	LR-7	-	LR-32	54.2	LR-57	16.1	LR-82	3.09	No-CAD 7	-
VHR-C-183	6.8	VHR-C-208	-	LR-8	-	LR-33	18.2	LR-58	-	LR-83	-	No-CAD 8	22.94245
VHR-C-184	4.07	VHR-C-209	2.35	LR-9	5.52	LR-34	3.15	LR-59	11.5	LR-84	2.31	No-CAD 9	-
VHR-C-185	-	VHR-C-210	5.45	LR-10	-	LR-35	-	LR-60	9.02	LR-85	16.3	No-CAD 10	19.7
VHR-C-186	3.14	VHR-C-211	-	LR-11	-	LR-36	6.15	LR-61	9.99	LR-86	-	No-CAD 11	-
VHR-C-187	-	VHR-C-212	3.28	LR-12	-	LR-37	6.57	LR-62	8.29	LR-87	4.65	No-CAD 12	-
VHR-C-188	-	VHR-C-213	39.6	LR-13	12.9	LR-38	-	LR-63	18.3	LR-88	-	No-CAD 13	0.898
VHR-C-189	8.6	VHR-C-214	23	LR-14	1.49	LR-39	-	LR-64	29.6	LR-89	-	No-CAD 14	21.4
VHR-C-190	-	VHR-C-215	-	LR-15	1.31	LR-40	-	LR-65	-	LR-90	-	No-CAD 15	-
VHR-C-191	11.7	VHR-C-216	1.69	LR-16	6.71	LR-41	21.1	LR-66	1.45	LR-91	-	No-CAD 16	-
VHR-C-192	12.2	VHR-C-217	-	LR-17	15	LR-42	-	LR-67	-	LR-92	1.7	No-CAD 17	-
VHR-C-193	-	VHR-C-218	-	LR-18	2.18	LR-43	-	LR-68	4.77	LR-93	14.5	No-CAD 18	5.58
VHR-C-194	-	VHR-C-219	-	LR-19	-	LR-44	42.6	LR-69	35.4	LR-94	3.89	No-CAD 19	-
VHR-C-195	34.9	VHR-C-220	2.21	LR-20	-	LR-45	-	LR-70	-	LR-95	7.63	No-CAD 20	7.43
VHR-C-196	-	VHR-C-221	3.52	LR-21	-	LR-46	-	LR-71	11.6	LR-96	2.8	No-CAD 21	-
VHR-C-197	13.8			LR-22	11	LR-47	5.2	LR-72	3.62	LR-97	5.01	No-CAD 22	0.864
VHR-C-198	-			LR-23	-	LR-48	-	LR-73	1.45	LR-98	0.318	No-CAD 23	38.9
VHR-C-199	-			LR-24	-	LR-49	32	LR-74	49.3	LR-99	1.42		
VHR-C-200	-			LR-25	-	LR-50	-	LR-75	5.38	LR-100	1.46		
Intra-assay CV for TACE protein measurment by ELISA (n=162) = Average of all CV values (%) = 11.91													

Missing values refer to TACE plasma protein levels that were below the limit of detection of the assay.

2.6.2 Method for measuring tumour factor necrosis alpha converting enzyme (TACE) protein levels in preparations of extracted proteins by ELISA - Human TACE ELISA Kit - RAB0003 SIGMA, (Missouri, USA)

Total protein levels measured by BCA in samples stored in MPER® were normalised. A volume of extracted protein was used to obtain a final concentration of 5000 ug/ml of total proteins (5 ug/ul) and diluted in assay/sample diluent buffer (Buffer C) accordingly. The following formula was used:

Initial Concentration x Initial Volume = Final concentration x Final volume

Concentration calculated by the BCA assay x volume to use = 5 ug/ul x 400 ul

$$\text{Volume to use (ul)} = \frac{5 \text{ ug/ul x } 400 \text{ ul}}{\text{Concentration calculated by the BCA assay}}$$

The total volume used for the ELISA was 400 ul and samples were run in triplicates (100 ul per well). Samples were not diluted in that case and the result was relative to the same amount of total proteins used for all the samples (5000 ug/ml or 5 ug/ul).

The TACE ELISA procedure was followed as in **section 2.6.1**.

***Table 5:** Intra-assay coefficient of variation (CV) of TACE ELISA for the measurement of cell membrane bound TACE protein levels*

Patient ID	%CV	Patient ID	%CV	Patient ID	%CV	Patient ID	%CV
VHR-C-001	4.02	VHR-C-006	3.47	VHR-C-033	n/a	LR-3	4.66
VHR-C-002	3.56	VHR-C-011	2.41	VHR-C-034	1.07	LR-4	3.64
VHR-C-003	0.199	VHR-C-012	1.12	VHR-C-056	3.15	LR-5	1.06
VHR-C-005	1.33	VHR-C-013	6.45	VHR-C-048	29.2	LR-6	7.59
VHR-C-008	1.37	VHR-C-014	22.3	VHR-C-055	13.2	LR-7	7.67
VHR-C-009	4.34	VHR-C-016	2.1	LR-12	6.07	LR-8	28.2
VHR-C-010	4.94	VHR-C-017	7.06	LR-13	9.87	LR-9	10.4
VHR-C-015	3.61	VHR-C-018	8.68	LR-14	16.7	LR-10	5.68
VHR-C-045	1.43	VHR-C-022	0.264	LR-15	3.74	LR-11	20.1
VHR-C-046	4.79	VHR-C-023	4.89	LR-16	4.03	LR-19	6.27
VHR-C-032	3.16	LR-2	48.4	LR-18	2.38	LR-20	26.7
Intra-assay CV for TACE protein measurment by ELISA (n=43) = Average of all CV values (%) = 8.17%							

2.7 Soluble TNFR1 ELISA

2.7.1 Method for measuring soluble tumour necrosis factor receptor 1 sTNFR1/TNFRSF1A protein levels in the plasma by ELISA – Human TNF RI/TNFRSF1A Immunoassay R&D - DRT100 (Minneapolis, USA)

TNFR1 ELISA was performed according to the manufacturer's instructions. Plasma samples were diluted 10-fold in Calibrator Diluent RD6O - 895120 (80 ul + 720 ul). The 8 standards were prepared by serial dilution in the same Calibrator Diluent RD6O with 500 pg/ml as the highest standard and only using the calibrator diluent for the lowest standard concentration (500 pg/ml; 250 pg/ml; 125 pg/ml; 62.5 pg/ml; 31.2 pg/ml; 15.6 pg/ml; 7.8 pg/ml; 0 pg/ml). All reagents were at room temperature before starting the experiment (which was between 22◦ and 28◦C).

A volume of 50 ul of Assay Diluent HD1-7 (895160) was added to each well. Samples were run in triplicate and 200 ul of diluted sample or prepared standard were added per well. Wells were covered and incubated for 2 hours at room temperature (which was between 22◦C and 28◦C). Wells were washed three times with the prepared wash buffer (20 ml of concentrated wash buffer with 480 ml of deionised water) and the plate was blotted against paper towels to remove any remaining wash buffer. A volume of 200 µL of sTNFRI Conjugate (890118) was added to each well. Wells were covered with a new adhesive strip and the plate was incubated for 2 hours at room temperature. Wells were washed three times with the prepared wash buffer and blot plate against paper towels to remove any remaining wash buffer. A volume of 200 µL of prepared Substrate Solution to each well (10 ml of color reagent A (895000) + 10 ml of color reagent B

(895001). The plate was incubated for 20 minutes at room temperature and protected from light.

A volume of 50 µL of Stop Solution (895032) was added to each well and the plate was gently tapped to ensure thorough mixing. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm with a wavelength correction set to 540 nm. For result analysis, a wavelength correction was made by subtracting all the readings at 540 nm from the readings at 450 nm. This subtraction corrected for optical imperfections in the plate. The average zero standard optical density was subtracted from all the readings. Then, the triplicate readings were averaged for each standard and sample.

A standard curve was created from the standards readings using a 4-parametric logistic regression curve. The sample concentration was then calculated after multiplying by the dilution factor. For creating the standard curve and calculating the sample concentrations, the following website was used: https://www.myassays.com/. The average of the minimum detectable concentration provided by the ELISA kit was 0.77 pg/mL. Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again.

TNFR1 ELISA intra-assay CV = 4.2 %

TNFR1 ELISA inter-assay CV = 8.62 %

<u>Table 6:</u> Inter-assay and intra-assay coefficient of variation (CV) of TNFR1 ELISA for the measurement of TNFR1 plasma protein levels

Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)
VHR-C-001	10.8	VHR-C-026	1.77	VHR-C-051	2.29	VHR-C-076	2.04	VHR-C-101	5.98	VHR-C-126	2.17	VHR-C-151	2.97
VHR-C-002	5.03	VHR-C-027	2.79	VHR-C-052	1.97	VHR-C-077	2.13	VHR-C-102	10.9	VHR-C-127	7.32	VHR-C-152	3.99
VHR-C-003	1.76	VHR-C-028	2.66	VHR-C-053	3.41	VHR-C-078	7.87	VHR-C-103	2.97	VHR-C-128	4.45	VHR-C-153	5.9
VHR-C-004	2.92	VHR-C-029	2.63	VHR-C-054	8	VHR-C-079	4.19	VHR-C-104	0.666	VHR-C-129	6.25	VHR-C-154	4.99
VHR-C-005	5.18	VHR-C-030	9.47	VHR-C-055	3.89	VHR-C-080	2.22	VHR-C-105	11.5	VHR-C-130	5.77	VHR-C-155	0.915
VHR-C-006	5.12	VHR-C-031	2.76	VHR-C-056	3.7	VHR-C-081	5.03	VHR-C-106	1.82	VHR-C-131	4.56	VHR-C-156	5.39
VHR-C-007	1.87	VHR-C-032	4.04	VHR-C-057	3.61	VHR-C-082	10.9	VHR-C-107	3.83	VHR-C-132	3.35	VHR-C-157	6.94
VHR-C-008	0.747	VHR-C-033	7.72	VHR-C-058	2.69	VHR-C-083	2.64	VHR-C-108	2.02	VHR-C-133	3.63	VHR-C-158	5.29
VHR-C-009	4.14	VHR-C-034	0.921	VHR-C-059	2.05	VHR-C-084	2.97	VHR-C-109	7.55	VHR-C-134	2.44	VHR-C-159	1.11
VHR-C-010	7.63	VHR-C-035	1.54	VHR-C-060	1.21	VHR-C-085	2.9	VHR-C-110	3.17	VHR-C-135	2.08	VHR-C-160	1.59
VHR-C-011	2.07	VHR-C-036	3.29	VHR-C-061	2.26	VHR-C-086	2.31	VHR-C-111	2.9	VHR-C-136	2.5	VHR-C-161	3.38
VHR-C-012	2.74	VHR-C-037	8.39	VHR-C-062	2.18	VHR-C-087	4.78	VHR-C-112	1.4	VHR-C-137	4	VHR-C-162	2.51
VHR-C-013	4.07	VHR-C-038	2.11	VHR-C-063	3.63	VHR-C-088	1.08	VHR-C-113	2	VHR-C-138	3.03	VHR-C-163	1.98
VHR-C-014	8.36	VHR-C-039	1.86	VHR-C-064	6.96	VHR-C-089	1.15	VHR-C-114	0.182	VHR-C-139	4.17	VHR-C-164	1.22
VHR-C-015	7.84	VHR-C-040	4.39	VHR-C-065	6.74	VHR-C-090	4.9	VHR-C-115	1.57	VHR-C-140	8.46	VHR-C-165	4.14
VHR-C-016	9.93	VHR-C-041	0.15	VHR-C-066	5.84	VHR-C-091	4.16	VHR-C-116	4.25	VHR-C-141	1.18	VHR-C-166	4.12
VHR-C-017	4.47	VHR-C-042	1.81	VHR-C-067	0.935	VHR-C-092	5.42	VHR-C-117	1.19	VHR-C-142	4.22	VHR-C-167	0.422
VHR-C-018	5.52	VHR-C-043	1.71	VHR-C-068	4.37	VHR-C-093	1.17	VHR-C-118	7.6	VHR-C-143	6.18	VHR-C-168	3.05
VHR-C-019	9.11	VHR-C-044	3.9	VHR-C-069	7.74	VHR-C-094	1.48	VHR-C-119	6	VHR-C-144	3.67	VHR-C-169	3.58
VHR-C-020	3.07	VHR-C-045	1.8	VHR-C-070	6.17	VHR-C-095	5.38	VHR-C-120	3.64	VHR-C-145	3.51	VHR-C-170	3.2
VHR-C-021	5.12	VHR-C-046	3.71	VHR-C-071	1.73	VHR-C-096	4.46	VHR-C-121	7.15	VHR-C-146	6.03	VHR-C-171	2
VHR-C-022	8.69	VHR-C-047	3.65	VHR-C-072	1.08	VHR-C-097	3.94	VHR-C-122	2.64	VHR-C-147	2.61	VHR-C-172	1.49
VHR-C-023	3.72	VHR-C-048	4.44	VHR-C-073	4.66	VHR-C-098	9.76	VHR-C-123	2.55	VHR-C-148	5.65	VHR-C-173	0.338
VHR-C-024	3.42	VHR-C-049	4.63	VHR-C-074	6.6	VHR-C-099	8.35	VHR-C-124	1.24	VHR-C-149	3	VHR-C-174	1.95
VHR-C-025	1.8	VHR-C-050	0.726	VHR-C-075	6.07	VHR-C-100	5.3	VHR-C-125	12.5	VHR-C-150	3.47	VHR-C-175	1.5
Patient ID	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**
VHR-C-176	1.94	VHR-C-201	1.87	LR-1	6.67	LR-26	2.1	LR-51	5.9	LR-76	9.25	No-CAD 1	2.78
VHR-C-177	2.58	VHR-C-202	1.2	LR-2	12.6	LR-27	0.407	LR-52	3.33	LR-77	0.231	No-CAD 2	0.53
VHR-C-178	6.66	VHR-C-203	6.3	LR-3	2.86	LR-28	2.61	LR-53	7.01	LR-78	2.72	No-CAD 3	2.42
VHR-C-179	3.9	VHR-C-204	6.03	LR-4	5.52	LR-29	0.565	LR-54	5.13	LR-79	3.88	No-CAD 4	0.782
VHR-C-180	0.947	VHR-C-205	3.89	LR-5	1.47	LR-30	6.11	LR-55	16.2	LR-80	2.5	No-CAD 5	5.2
VHR-C-181	3.07	VHR-C-206	2.74	LR-6	0.767	LR-31	1.41	LR-56	10.8	LR-81	5.59	No-CAD 6	2.49
VHR-C-182	2.81	VHR-C-207	5.15	LR-7	2.96	LR-32	5.09	LR-57	7.67	LR-82	0.175	No-CAD 7	1.02
VHR-C-183	3.82	VHR-C-208	0.335	LR-8	4.12	LR-33	2.92	LR-58	1.76	LR-83	3	No-CAD 8	7.64
VHR-C-184	2.42	VHR-C-209	2.12	LR-9	8.18	LR-34	8.33	LR-59	4.19	LR-84	2.64	No-CAD 9	1.32
VHR-C-185	3.35	VHR-C-210	7.3	LR-10	4.28	LR-35	6.43	LR-60	1.28	LR-85	2.39	No-CAD 10	9.34
VHR-C-186	0.993	VHR-C-211	4.12	LR-11	2.15	LR-36	2.62	LR-61	5.38	LR-86	1.64	No-CAD 11	1.24
VHR-C-187	1.31	VHR-C-212	3.28	LR-12	4.89	LR-37	2.84	LR-62	5.92	LR-87	4.5	No-CAD 12	4.1
VHR-C-188	4.04	VHR-C-213	3.55	LR-13	2.65	LR-38	3.86	LR-63	5.37	LR-88	8.01	No-CAD 13	1.43
VHR-C-189	2.9	VHR-C-214	3.97	LR-14	3.12	LR-39	1.89	LR-64	9.88	LR-89	13.6	No-CAD 14	1.26
VHR-C-190	2.97	VHR-C-215	9.56	LR-15	0.953	LR-40	9.72	LR-65	5.56	LR-90	5.36	No-CAD 15	14.7
VHR-C-191	4.92	VHR-C-216	6.66	LR-16	1.96	LR-41	8.16	LR-66	3.62	LR-91	7.34	No-CAD 16	5.54
VHR-C-192	2.83	VHR-C-217	10.3	LR-17	1.26	LR-42	11.8	LR-67	5	LR-92	7.21	No-CAD 17	5.29
VHR-C-193	5.08	VHR-C-218	1.76	LR-18	6.89	LR-43	5.02	LR-68	3.74	LR-93	22	No-CAD 18	11.9
VHR-C-194	4.52	VHR-C-219	6.5	LR-19	1.23	LR-44	7	LR-69	8.13	LR-94	0.969	No-CAD 19	1.35
VHR-C-195	1.46	VHR-C-220	5.61	LR-20	3.41	LR-45	2.66	LR-70	7.06	LR-95	9.23	No-CAD 20	3.21
VHR-C-196	0.84	VHR-C-221	4.65	LR-21	0.4	LR-46	5.9	LR-71	3.83	LR-96	2.84	No-CAD 21	1.83
VHR-C-197	3.99			LR-22	0.73	LR-47	4.82	LR-72	2.81	LR-97	2.86	No-CAD 22	6.32
VHR-C-198	1.29			LR-23	4.65	LR-48	6.36	LR-73	3.93	LR-98	0.449	No-CAD 23	3.01
VHR-C-199	5.81			LR-24	3.51	LR-49	3.38	LR-74	8.27	LR-99	2.13		
VHR-C-200	2.49			LR-25	3.23	LR-50	5.53	LR-75	8.05	LR-100	3.02		
Intra-assay CV for sTNFR1 protein measurment by ELISA (n=344) = Average of all CV values (%) = 4.2													

	Measurment 1 mean - (pg/ml)	CV (%)	Measurment 2 mean- (pg/ml)	CV (%)	Mean of means	StD of Means	CV (%) of Means
VHR-C-084	3005	2.97	3583	4.98	3294	408.7077	12.40764176
VHR-C-085	917.1	2.9	982.1	8.15	949.6	45.96194	4.840136982
Inter-assay CV (%)							**8.62388937**

2.7.2 Method for measuring soluble tumour necrosis factor receptor 1 sTNFR1/TNFRSF1A protein levels in preparations of extracted proteins by ELISA - Human TNF RI/TNFRSF1A Immunoassay R&D - DRT100 (Minneapolis, USA)

Total protein levels measured by BCA in samples stored in MPER® were normalised. A volume of extracted protein was used to obtain a final concentration of 5000 ug/ml of total proteins (5 ug/ul) and diluted in Calibrator Diluent RD6O accordingly. The following formula was used:

Initial Concentration x Initial Volume = Final concentration x Final volume

Concentration calculated by the BCA assay x volume to use = 5 ug/ul x 400 ul

$$\text{Volume to use (ul)} = \frac{5\ \text{ug/ul x}\ 400\ \text{ul}}{\text{Concentration calculated by the BCA assay}}$$

The volume was made up to 800 ul using the Calibrator Diluent RD6O and samples were run in triplicates (200 ul per well). Samples were not diluted in that case and the result was relative to the same amount of proteins used for all the samples (5000 ug/ml or 5 ug/ul). The TNFR1 ELISA procedure was followed as in **section 2.7.1.**

***Table 7:** Intra-assay coefficient of variation (CV) of TNFR1 ELISA for the measurement of cell membrane bound TNFR1 protein levels*

Sample ID	CV(%)	Sample ID	CV(%)
VHR-C-015	1.61	LR-8	1.35
VHR-C-016	1.72	LR-9	10.3
VHR-C-018	5.84	LR-10	7.61
VHR-C-011	10.5	LR-11	9.44
VHR-C-012	4.36	LR-12	8.75
VHR-C-013	5.98	LR-13	5.08
VHR-C-014	7.93	LR-14	8.2
Intra-assay CV (%) = 6.33			

2.8 Soluble TNFR2 ELISA

2.8.1 Method for measuring soluble tumour necrosis factor receptor 2 sTNFR2/TNFRSF1B in the plasma by ELISA – Human TNF RII/TNFRSF1B Immunoassay R&D - DRT200 (Minneapolis, USA)

TNFR2 ELISA was performed according to the manufacturer's instructions. Plasma samples were diluted 10-fold in Calibrator Diluent RD6O - 895120 (80 ul + 720 ul). The 8 standards were prepared by serial dilution in the same Calibrator Diluent RD6O with 500 pg/ml as the highest standard and only using the calibrator diluent for the lowest standard concentration (500 pg/ml; 250 pg/ml; 125 pg/ml; 62.5 pg/ml; 31.2 pg/ml; 15.6 pg/ml; 7.8 pg/ml; 0 pg/ml).

A volume of 50 ul of Assay Diluent RD1-6 (895158) was added to each well. Samples were run in triplicate and 200 ul of diluted sample or prepared standard were added per well. Wells were covered and incubated for 2 hours at room temperature. Wells were washed three times with the prepared wash buffer (20 ml of concentrated wash buffer with 480 ml of deionised water) and the plate blotted against paper towels to remove any remaining wash buffer. A volume of 200 µL of sTNFRII Conjugate (890122) was added to each well. Wells were covered with a new adhesive strip and the plate was incubated for 2 hours at room temperature. Wells were washed three times with the prepared wash buffer and blot plate against paper towels to remove any remaining wash buffer. A volume of 200 uL of prepared Substrate Solution to each well (10 ml of color reagent A (895000) + 10 ml of color reagent B (895001). The plate was incubated for 20 minutes at room temperature and protected from light. A volume of 50 uL of

Stop Solution (895032) was added to each well and the plate was gently tapped to ensure thorough mixing. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm with a wavelength correction set to 540 nm. For result analysis, a wavelength correction was made by subtracting all the readings at 540 nm from the readings at 450 nm. This subtraction corrected for optical imperfections in the plate. The average zero standard optical density was subtracted from all the readings. Then, the triplicate readings were averaged for each standard and sample. A standard curve was created from the standards readings using a 4-parametric logistic regression curve. The sample concentration was then calculated after multiplying by the dilution factor. For creating the standard curve and calculating the sample concentrations, the following website was used: https://www.myassays.com/. The average of the minimum detectable concentration provided by the kit was 0.6 pg/mL. Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again. A new standard curve was made for each plate.

TNFR2 ELISA intra-assay CV = 4.2 %

TNFR2 ELISA inter-assay CV = 5.07%

<u>Table 8:</u> Inter-assay and intra-assay coefficient of variation (CV) of TNFR2 ELISA for the measurement of TNFR2 plasma protein levels

Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)
VHR-C-001	3.09	VHR-C-026	9.55	VHR-C-051	1.09	VHR-C-076	0.513	VHR-C-101	2.1	VHR-C-126	1.96	VHR-C-151	2.83
VHR-C-002	5.13	VHR-C-027	2.43	VHR-C-052	6	VHR-C-077	2.31	VHR-C-102	3.4	VHR-C-127	2.79	VHR-C-152	5.49
VHR-C-003	3.35	VHR-C-028	9.99	VHR-C-053	3.02	VHR-C-078	4.42	VHR-C-103	3.49	VHR-C-128	3.99	VHR-C-153	0.663
VHR-C-004	3.32	VHR-C-029	0.511	VHR-C-054	3.41	VHR-C-079	0.738	VHR-C-104	3.74	VHR-C-129	2.54	VHR-C-154	2.64
VHR-C-005	1.83	VHR-C-030	2.63	VHR-C-055	4.18	VHR-C-080	4.33	VHR-C-105	3.33	VHR-C-130	5.12	VHR-C-155	0.796
VHR-C-006	0.811	VHR-C-031	2.45	VHR-C-056	1.49	VHR-C-081	4.21	VHR-C-106	6.64	VHR-C-131	7.66	VHR-C-156	8.29
VHR-C-007	9.28	VHR-C-032	0.92	VHR-C-057	1.65	VHR-C-082	4.26	VHR-C-107	3.55	VHR-C-132	7.41	VHR-C-157	4.64
VHR-C-008	3.37	VHR-C-033	3.57	VHR-C-058	5.31	VHR-C-083	2.64	VHR-C-108	2.12	VHR-C-133	1.82	VHR-C-158	3.36
VHR-C-009	1.99	VHR-C-034	1.22	VHR-C-059	3.61	VHR-C-084	6.52	VHR-C-109	1.62	VHR-C-134	1.11	VHR-C-159	2.57
VHR-C-010	4.38	VHR-C-035	4.3	VHR-C-060	4.15	VHR-C-085	1.27	VHR-C-110	4.94	VHR-C-135	6.06	VHR-C-160	1.18
VHR-C-011	21.5	VHR-C-036	3.15	VHR-C-061	2.72	VHR-C-086	2.28	VHR-C-111	1.61	VHR-C-136	3.55	VHR-C-161	2.48
VHR-C-012	4.67	VHR-C-037	1.35	VHR-C-062	5.05	VHR-C-087	1.95	VHR-C-112	3.19	VHR-C-137	7.29	VHR-C-162	11
VHR-C-013	2.52	VHR-C-038	6.55	VHR-C-063	4.61	VHR-C-088	1.14	VHR-C-113	1.67	VHR-C-138	10.3	VHR-C-163	3.74
VHR-C-014	2.01	VHR-C-039	2.16	VHR-C-064	13.3	VHR-C-089	3.45	VHR-C-114	8.41	VHR-C-139	4.73	VHR-C-164	5.48
VHR-C-015	3.09	VHR-C-040	1.46	VHR-C-065	6.69	VHR-C-090	8.15	VHR-C-115	1.57	VHR-C-140	1.06	VHR-C-165	4.03
VHR-C-016	4.14	VHR-C-041	4.56	VHR-C-066	4.89	VHR-C-091	5.21	VHR-C-116	2.03	VHR-C-141	0.771	VHR-C-166	1.94
VHR-C-017	4.96	VHR-C-042	6.29	VHR-C-067	16.5	VHR-C-092	6.14	VHR-C-117	2.68	VHR-C-142	1	VHR-C-167	5.47
VHR-C-018	2.42	VHR-C-043	3.72	VHR-C-068	9.94	VHR-C-093	6.31	VHR-C-118	4.06	VHR-C-143	3.35	VHR-C-168	2.04
VHR-C-019	3.63	VHR-C-044	11.1	VHR-C-069	5.53	VHR-C-094	3.96	VHR-C-119	4.27	VHR-C-144	3.62	VHR-C-169	1.57
VHR-C-020	2.66	VHR-C-045	6.1	VHR-C-070	0.0308	VHR-C-095	5.56	VHR-C-120	2.63	VHR-C-145	0.721	VHR-C-170	3.83
VHR-C-021	12.7	VHR-C-046	2.63	VHR-C-071	4.73	VHR-C-096	0.707	VHR-C-121	2.69	VHR-C-146	3.5	VHR-C-171	5.55
VHR-C-022	5.83	VHR-C-047	6.61	VHR-C-072	4.38	VHR-C-097	1.12	VHR-C-122	1.77	VHR-C-147	4.97	VHR-C-172	12
VHR-C-023	5.28	VHR-C-048	1.89	VHR-C-073	2.33	VHR-C-098	4.88	VHR-C-123	3.5	VHR-C-148	3.58	VHR-C-173	2.31
VHR-C-024	3.36	VHR-C-049	5.64	VHR-C-074	7.12	VHR-C-099	5.61	VHR-C-124	1.86	VHR-C-149	6.19	VHR-C-174	4.19
VHR-C-025	7.94	VHR-C-050	1.13	VHR-C-075	2.62	VHR-C-100	1.13	VHR-C-125	4.22	VHR-C-150	3.66	VHR-C-175	3.83
Patient ID	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**
VHR-C-176	2.5	VHR-C-201	5.56	LR-1	1.52	LR-26	4.61	LR-51	3.82	LR-76	4.3	No-CAD 1	4.85
VHR-C-177	2.91	VHR-C-202	10.4	LR-2	1.67	LR-27	1.34	LR-52	2.11	LR-77	5.56	No-CAD 2	3.14
VHR-C-178	4.59	VHR-C-203	2.13	LR-3	3.79	LR-28	6.48	LR-53	7.57	LR-78	6.64	No-CAD 3	2.14
VHR-C-179	3.79	VHR-C-204	3.68	LR-4	1.8	LR-29	3.79	LR-54	2.35	LR-79	9.54	No-CAD 4	2.54
VHR-C-180	4.41	VHR-C-205	7.66	LR-5	3.88	LR-30	3.43	LR-55	8.15	LR-80	4.92	No-CAD 5	0.385
VHR-C-181	4.46	VHR-C-206	2.83	LR-6	4.09	LR-31	2.78	LR-56	5.95	LR-81	2.08	No-CAD 6	12.8
VHR-C-182	0.656	VHR-C-207	3.76	LR-7	3.11	LR-32	8.82	LR-57	0.872	LR-82	3.38	No-CAD 7	6.25
VHR-C-183	3.75	VHR-C-208	7.3	LR-8	5.32	LR-33	7.61	LR-58	6.53	LR-83	2.13	No-CAD 8	2.19
VHR-C-184	1.76	VHR-C-209	3.68	LR-9	2.11	LR-34	2.98	LR-59	5.63	LR-84	1.54	No-CAD 9	1.73
VHR-C-185	1.86	VHR-C-210	4.08	LR-10	0.591	LR-35	6.92	LR-60	5.31	LR-85	0.832	No-CAD 10	3.29
VHR-C-186	2.04	VHR-C-211	2.05	LR-11	0.366	LR-36	7.99	LR-61	5.47	LR-86	12.4	No-CAD 11	1.92
VHR-C-187	2.06	VHR-C-212	8.33	LR-12	2.98	LR-37	0.497	LR-62	5.16	LR-87	2.11	No-CAD 12	1.89
VHR-C-188	1.33	VHR-C-213	3.79	LR-13	3.85	LR-38	8.27	LR-63	8.53	LR-88	3.63	No-CAD 13	4.19
VHR-C-189	2.12	VHR-C-214	8.87	LR-14	3.83	LR-39	4.07	LR-64	3.18	LR-89	9.6	No-CAD 14	2.2
VHR-C-190	1.78	VHR-C-215	7.04	LR-15	11.4	LR-40	3.65	LR-65	3.2	LR-90	6.86	No-CAD 15	2.35
VHR-C-191	6.25	VHR-C-216	9.95	LR-16	0.677	LR-41	6.42	LR-66	4.38	LR-91	1.92	No-CAD 16	6.03
VHR-C-192	6.45	VHR-C-217	4.28	LR-17	7.21	LR-42	2.18	LR-67	4.39	LR-92	3.39	No-CAD 17	1.72
VHR-C-193	5.94	VHR-C-218	2.4	LR-18	0.476	LR-43	3.78	LR-68	2.21	LR-93	2.52	No-CAD 18	7.24
VHR-C-194	7.87	VHR-C-219	3.12	LR-19	7.03	LR-44	8.89	LR-69	11	LR-94	1.95	No-CAD 19	4
VHR-C-195	2.86	VHR-C-220	2.91	LR-20	7.65	LR-45	2.72	LR-70	11.9	LR-95	2.12	No-CAD 20	4.67
VHR-C-196	4.97	VHR-C-221	1.68	LR-21	3.3	LR-46	0.756	LR-71	4.57	LR-96	4.27	No-CAD 21	1.95
VHR-C-197	3.29			LR-22	4.54	LR-47	7.56	LR-72	7.71	LR-97	2.91	No-CAD 22	3.44
VHR-C-198	2.95			LR-23	1	LR-48	2.96	LR-73	8.28	LR-98	2.8	No-CAD 23	4.38
VHR-C-199	3.96			LR-24	1.89	LR-49	2.76	LR-74	6.93	LR-99	5.3		
VHR-C-200	5.09			LR-25	6.88	LR-50	9.53	LR-75	0.646	LR-100	0.598		
Intra-assay CV for sTNFR2 protein measurment by ELISA (n=344) = Average of all CV values (%) = 4.2													

	Measurment 1 mean - (pg/ml)	CV (%)	Measurment 2 mean- (pg/ml)	CV (%)	Mean of means	StD of Means	CV (%) of Means
VHR-C-122	3994	1.77	3608	1.35	3801	272.9432	7.180827
VHR-C-123	8398	3.5	8757	2.45	8577.5	253.8513	2.959503
Inter-assay CV (%)							**5.070165**

2.8.2 Method for measuring soluble tumour necrosis factor receptor 2 sTNFR2/TNFRSF1B protein levels in preparations of extracted proteins by ELISA - Human TNF RII/TNFRSF1B Immunoassay R&D - DRT200 (Minneapolis, USA)

Total protein levels measured by BCA in samples stored in MPER® were normalised. A volume of extracted protein was used to obtain a final concentration of 5000 ug/ml of total proteins (5 ug/ul) and diluted in Calibrator Diluent RD6O accordingly. The following formula was used:

Initial Concentration x Initial Volume = Final concentration x Final volume

Concentration calculated by the BCA assay x volume to use = 5 ug/ul x 400 ul

$$\text{Volume to use (ul)} = \frac{5\ \text{ug/ul x}\ 400\ \text{ul}}{\text{Concentration calculated by the BCA assay}}$$

The volume was made up to 800 ul using the Calibrator Diluent RD6O and samples were run in triplicates (200 ul per well). Samples were not diluted in that case and the result was relative to the same amount of proteins used for all the samples (5000 ug/ml or 5 ug/ul). The TNFR2 ELISA procedure was followed as in **section 2.8.1.**

<u>Table 9:</u> Intra-assay coefficient of variation (CV) of TNFR2 ELISA for the measurement of cell membrane bound TNFR2 protein levels

Sample ID	CV (%)	Sample ID	CV (%)
VHR-C-015	12.8	LR-8	4.31
VHR-C-016	1.15	LR-9	4.14
VHR-C-018	4.86	LR-10	4.07
VHR-C-011	3.57	LR-11	4.06
VHR-C-012	12.3	LR-12	2.16
VHR-C-013	6.4	LR-13	2.11
VHR-C-014	1.23	LR-14	2.09
Inter-assay CV (%) = 4.7			

2.9 TIMP3 ELISA

2.9.1 Method for measuring TIMP3 protein levels in the plasma by ELISA- TIMP3 (MIG-5) Human ELISA -abcam ab119608 (Cambridge, UK)

TIMP3 ELISA was performed according to the manufacturer's instructions. Samples were diluted 2-fold in the sample diluent buffer (200 ul of sample + 200 ul of diluent). The 6 standards were prepared by serial dilution in the same diluent with 10000 pg/ml as the highest standard and only using the assay/sample diluent buffer for the lowest standard concentration (10000 pg/ml; 5000 pg/ml; 2500 pg/ml; 1250 pg/ml; 625 pg/ml; 0 pg/ml).

Samples were run in triplicate and 200 ul of diluted sample or prepared standard were added per well. Wells were covered and incubated at 37°C for 90 minutes. The cover was removed and the contents of each well were discard contents and the plate was blotted onto paper towels. Wells were not left to dry at any time. A volume of 100 uL of prepared 1X Biotinylated Anti-Human TIMP3 antibody (diluted 100-fold with the Antibody Diluent Buffer) were added into each well and the plate was incubated at 37°C for 60 minutes. The plate was washed three times with 0.01M PBS and each time the washing buffer was left to stay in the wells for one minute. The washing buffer was then discarded and the plate was blotted onto paper towels. A volume of 100 uL of prepared 1X Avidin-Biotin-Peroxidase Complex (ABC) working solution (diluted 100-fold with ABC Diluent Buffer) was added into each well and incubate the plate at 37°C for 30 minutes. The plate was washed five times with 0.01M PBS and each time the washing buffer was left to stay in the wells for one minute. The washing buffer was

then discarded and the plate was blotted onto paper towels. A volume of 90 uL of TMB color developing agent was added into each well and incubate plate at 37°C in dark for 20 - 25 minutes. A volume of 100 uL of TMB Stop Solution was added into each well. The color changed into yellow immediately. The optical density absorbance of the plate was read at 540 nm in a microplate reader within 30 minutes after adding the stop solution. For the result analysis, the mean absorbance was calculated for each set of triplicate standards and samples. A standard curve was created from the standards using a 4-parametric logistic regression curve. The sample concentration was then calculated after multiplying by the dilution factor. For creating the standard curve and calculating the sample concentrations, the following website was used: https://www.myassays.com/. The limit of sensitivity provided by the kit was < 2pg/ml.

Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again. A new standard curve was made for each plate.

TIMP3 ELISA intra-assay CV = 5.5%

TIMP3 ELISA inter-assay CV = 26.6%

Note: To calculate the inter-assay CV, the tested samples were measured for the second time after a total of 1 year and 3 months. This questions the issue of TIMP3 degradation over time. For all of the patient samples, TIMP3 levels were measured within 6 months of blood collection and the intra-assay CV was 5.5%. This shows that TIMP3 protein measurement from a frozen plasma aliquot should take place as close as possible to the time of collection.

<u>Table 10:</u> Inter-assay and intra-assay coefficient of variation (CV) of TIMP3 ELISA for the measurement of TIMP3 plasma protein levels

Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	CV (%)	Patient ID	
VHR-C-001	4.76	VHR-C-026	1.27	VHR-C-051	5.71	VHR-C-076	8.28	VHR-C-101	3.35	VHR-C-126	1.33	VHR-C-151	1.64
VHR-C-002	8.19	VHR-C-027	2.34	VHR-C-052	2.37	VHR-C-077	0.574	VHR-C-102	0.347	VHR-C-127	1.65	VHR-C-152	1.51
VHR-C-003	1.61	VHR-C-028	2.29	VHR-C-053	9.99	VHR-C-078	1.85	VHR-C-103	1.65	VHR-C-128	5.3	VHR-C-153	3.3
VHR-C-004	1.73	VHR-C-029	10.7	VHR-C-054	39.2	VHR-C-079	35.6	VHR-C-104	2.11	VHR-C-129	2.11	VHR-C-154	6.47
VHR-C-005	16	VHR-C-030	28.9	VHR-C-055	3.07	VHR-C-080	5.72	VHR-C-105	2.43	VHR-C-130	4.94	VHR-C-155	2.45
VHR-C-006	4.72	VHR-C-031	5.92	VHR-C-056	2.75	VHR-C-081	5.32	VHR-C-106	1.55	VHR-C-131	3.58	VHR-C-156	9.12
VHR-C-007	2.41	VHR-C-032	3.54	VHR-C-057	2.84	VHR-C-082	9.72	VHR-C-107	1.98	VHR-C-132	10.6	VHR-C-157	16.7
VHR-C-008	3.77	VHR-C-033	4.64	VHR-C-058	0.396	VHR-C-083	5.12	VHR-C-108	3.05	VHR-C-133	5.91	VHR-C-158	6.62
VHR-C-009	3.06	VHR-C-034	6.12	VHR-C-059	2.64	VHR-C-084	3.96	VHR-C-109	2.1	VHR-C-134	1.37	VHR-C-159	7.47
VHR-C-010	1.79	VHR-C-035	3.21	VHR-C-060	4.09	VHR-C-085	6.63	VHR-C-110	1.5	VHR-C-135	3.13	VHR-C-160	4.92
VHR-C-011	1.79	VHR-C-036	62.8	VHR-C-061	3.22	VHR-C-086	2.32	VHR-C-111	6.94	VHR-C-136	8.67	VHR-C-161	4.37
VHR-C-012	2.61	VHR-C-037	58.8	VHR-C-062	4.08	VHR-C-087	2.11	VHR-C-112	3.2	VHR-C-137	4.58	VHR-C-162	15.7
VHR-C-013	2.45	VHR-C-038	1.14	VHR-C-063	4.22	VHR-C-088	10.3	VHR-C-113	8.57	VHR-C-138	4.64	VHR-C-163	10.5
VHR-C-014	0.586	VHR-C-039	3.16	VHR-C-064	1.96	VHR-C-089	4.05	VHR-C-114	12.6	VHR-C-139	2.91	VHR-C-164	4.23
VHR-C-015	6.75	VHR-C-040	4.49	VHR-C-065	2.34	VHR-C-090	3.19	VHR-C-115	4.35	VHR-C-140	0.0804	VHR-C-165	0.846
VHR-C-016	5.93	VHR-C-041	1.43	VHR-C-066	0.657	VHR-C-091	11.1	VHR-C-116	2.77	VHR-C-141	3.95	VHR-C-166	3.22
VHR-C-017	4.11	VHR-C-042	3.54	VHR-C-067	2.27	VHR-C-092	1.24	VHR-C-117	6.3	VHR-C-142	0	VHR-C-167	0.491
VHR-C-018	13.2	VHR-C-043	2.66	VHR-C-068	5.41	VHR-C-093	10.4	VHR-C-118	0.745	VHR-C-143	2.16	VHR-C-168	11.8
VHR-C-019	28.7	VHR-C-044	3.15	VHR-C-069	7.9	VHR-C-094	8.02	VHR-C-119	4.95	VHR-C-144	2.51	VHR-C-169	6.12
VHR-C-020	9.89	VHR-C-045	2.45	VHR-C-070	3.63	VHR-C-095	3.42	VHR-C-120	5.13	VHR-C-145	1.02	VHR-C-170	24.8
VHR-C-021	3.86	VHR-C-046	4.38	VHR-C-071	19.9	VHR-C-096	13.6	VHR-C-121	3.38	VHR-C-146	8	VHR-C-171	2.77
VHR-C-022	3	VHR-C-047	1.83	VHR-C-072	3.56	VHR-C-097	2.2	VHR-C-122	2.97	VHR-C-147	3.35	VHR-C-172	1.72
VHR-C-023	4.05	VHR-C-048	9.63	VHR-C-073	1.93	VHR-C-098	3.32	VHR-C-123	4.38	VHR-C-148	2.86	VHR-C-173	8.11
VHR-C-024	2.52	VHR-C-049	0.65	VHR-C-074	1.75	VHR-C-099	3.86	VHR-C-124	4.93	VHR-C-149	3.84	VHR-C-174	4.31
VHR-C-025	3.21	VHR-C-050	3.15	VHR-C-075	2.43	VHR-C-100	3.89	VHR-C-125	n/a	VHR-C-150	2.56	VHR-C-175	3.98
Patient ID	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	**CV (%)**	**Patient ID**	
VHR-C-176	3.33	VHR-C-201	5.02	LR-1	8.16	LR-26	1.97	LR-51	3.37	LR-76	1.59	No-CAD 1	0.737
VHR-C-177	1.04	VHR-C-202	6.78	LR-2	16.8	LR-27	3.83	LR-52	1.33	LR-77	17.8	No-CAD 2	8.32
VHR-C-178	5	VHR-C-203	4.7	LR-3	6.14	LR-28	4.64	LR-53	2.01	LR-78	4.35	No-CAD 3	6.71
VHR-C-179	3.95	VHR-C-204	3.83	LR-4	4.26	LR-29	1.6	LR-54	2.39	LR-79	2.99	No-CAD 4	3.26
VHR-C-180	1.15	VHR-C-205	4.68	LR-5	10.7	LR-30	2.49	LR-55	0.955	LR-80	3.48	No-CAD 5	3
VHR-C-181	0.796	VHR-C-206	2.27	LR-6	6	LR-31	5.75	LR-56	1.51	LR-81	10.3	No-CAD 6	2.13
VHR-C-182	2.68	VHR-C-207	5.09	LR-7	7.37	LR-32	4.17	LR-57	1.7	LR-82	5.98	No-CAD 7	0.794
VHR-C-183	1.46	VHR-C-208	13.7	LR-8	13.6	LR-33	4.25	LR-58	6.79	LR-83	3.91	No-CAD 8	8.37
VHR-C-184	9.73	VHR-C-209	5.44	LR-9	4.49	LR-34	6.98	LR-59	1.6	LR-84	5.04	No-CAD 9	2.46
VHR-C-185	18.1	VHR-C-210	7.09	LR-10	7.23	LR-35	6.02	LR-60	3.12	LR-85	4.97	No-CAD 10	1.6
VHR-C-186	3.09	VHR-C-211	3.28	LR-11	8.34	LR-36	6.27	LR-61	2.09	LR-86	0.698	No-CAD 11	1.14
VHR-C-187	2.69	VHR-C-212	2.92	LR-12	6.33	LR-37	3.9	LR-62	2.41	LR-87	2.72	No-CAD 12	2.85
VHR-C-188	0.22	VHR-C-213	3.46	LR-13	2.92	LR-38	5.01	LR-63	2.48	LR-88	4.86	No-CAD 13	6.33
VHR-C-189	5.02	VHR-C-214	7.76	LR-14	6.06	LR-39	9.09	LR-64	7.65	LR-89	1.94	No-CAD 14	5.4
VHR-C-190	8.3	VHR-C-215	3.05	LR-15	8.3	LR-40	6.21	LR-65	0.399	LR-90	5.66	No-CAD 15	2.85
VHR-C-191	4.02	VHR-C-216	10.3	LR-16	2.79	LR-41	12.3	LR-66	3.05	LR-91	2.64	No-CAD 16	11.1
VHR-C-192	4.11	VHR-C-217	8.47	LR-17	2.73	LR-42	8.66	LR-67	5.86	LR-92	0.833	No-CAD 17	8.21
VHR-C-193	5.83	VHR-C-218	8.35	LR-18	1.44	LR-43	5.36	LR-68	2.41	LR-93	4.19	No-CAD 18	4.48
VHR-C-194	1.29	VHR-C-219	3.08	LR-19	2.17	LR-44	5.91	LR-69	1.55	LR-94	5.26	No-CAD 19	5.41
VHR-C-195	6.82	VHR-C-220	13.2	LR-20	3.44	LR-45	5.77	LR-70	2.71	LR-95	4.68	No-CAD 20	4.55
VHR-C-196	5.47	VHR-C-221	6.93	LR-21	3	LR-46	11.8	LR-71	4.57	LR-96	2.99	No-CAD 21	6.03
VHR-C-197	5.9			LR-22	2.77	LR-47	3.34	LR-72	4.76	LR-97	12.6	No-CAD 22	0.856
VHR-C-198	5.61			LR-23	5.66	LR-48	20.3	LR-73	4.62	LR-98	1.6	No-CAD 23	15.8
VHR-C-199	8.91			LR-24	14.7	LR-49	2.05	LR-74	5.57	LR-99	4.19		
VHR-C-200	3.86			LR-25	1.79	LR-50	2.15	LR-75	2.54	LR-100	1.21		
Intra-assay CV for sTNFR1 protein measurment by ELISA (n=343) = Average of all CV values (%) = 5.49													

	Measurment 1 mean - (pg/ml)	CV (%)	Measurment 2 mean- (pg/ml)	CV (%)	Mean of means	StD of Means	CV (%) of Means
VHR-C-046	1628	4.38	934.2	12.8	1281.1	490.5907	38.29449
VHR-C-049	8498	0.65	6882	3.27	7690.0	1142.685	14.85936
Inter-assay CV (%)							**26.57692**

2.9.2 Method for measuring TIMP3 protein levels in preparations of extracted proteins by ELISA - TIMP3 (MIG-5) Human ELISA -abcam ab119608 (Cambridge, UK)

Total protein levels measured by BCA in samples stored in MPER® were normalised. A volume of extracted protein was used to obtain a final concentration of 5000 ug/ml of total proteins (5 ug/ul) and diluted in the assay diluent accordingly. The following formula was used:

Initial Concentration x Initial Volume = Final concentration x Final volume

Concentration calculated by the BCA assay x volume to use = 5 ug/ul x 400 ul

$$\text{Volume to use (ul)} = \frac{5\ \text{ug/ul x}\ 400\ \text{ul}}{\text{Concentration calculated by the BCA assay}}$$

The volume was made up to 400 ul using the assay diluent and samples were run in triplicates (100 ul per well). Samples were not diluted in that case and the result was relative to the same amount of proteins used for all the samples (5000 ug/ml or 5 ug/ul). The TIMP3 ELISA procedure was followed as in **section 2.9.1.**

***Table 11:** Intra-assay CV (%) of TIMP3 ELISA for cell bound TIMP3*

Patient ID	CV (%)	Patient ID	CV (%)
VHR-C-008	4.44	LR-8	0
VHR-C-009	4.73	LR-9	5.66
VHR-C-010	55	LR-10	12.2
VHR-C-011	13.9	LR-11	5.08
VHR-C-012	2.84	LR-14	10.3
VHR-C-013	5.28	LR-15	0
VHR-C-014	12.2	VHR-C-002	4.91
VHR-C-015	3.99	VHR-C-003	6.33
LR-6	14.3	LR-12	7.6
LR-7	4.64	LR-13	4.04
Inter-assay CV (%) = 8.87			

2.10 Method for Meso Scale ELISA - Proinflammatory Panel 1 (human) Kit – IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α - MSD® MULTI-SPOT Assay System - K15049D (Maryland, USA)

MSD measures the levels of protein targets within a single, small-volume sample. The assays in the Proinflammatory Panel 1 (human) are sandwich immunoassays. The provided MSD plates are pre-coated with capture antibodies on independent and well-defined spots, as shown in the layout below. Multiplex assays are provided on 10-spot MULTI-SPOT® plates individual assays. The sample is added in each well and a solution containing detection antibodies conjugated to electrochemiluminescent labels (MSD SULFO-TAGTM) over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The MSD buffer is then added and creates the appropriate chemical environment for electrochemiluminescence (ECL). The plate is then loaded into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample. V-PLEX assay kits have been validated according to the principles outlined in “Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement” by J. W. Lee, et al (159).

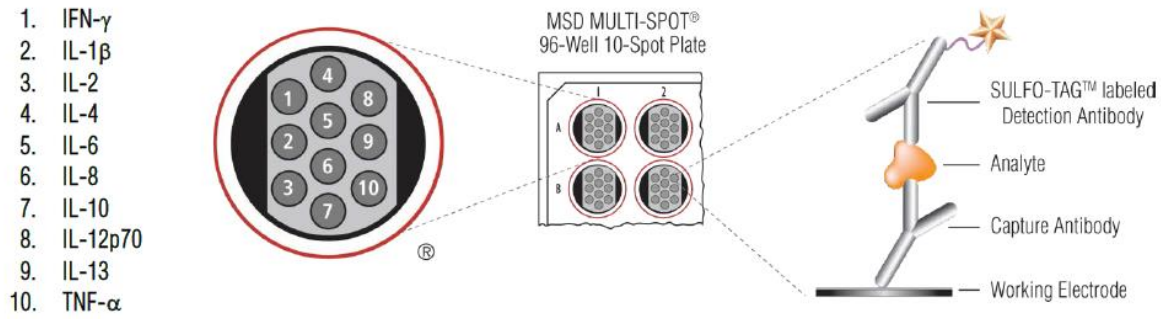


***Figure 6:* Multiplex plate spot diagram showing placement of analyte capture antibodies.**

Procedure:

The procedure was followed according to the manufacturer's instructions. Samples were diluted 2-fold using Diluent 2 (R51BB-3) (25 ul of sample + 25 ul of diluent). A total of 8 standards/calibrators were prepared by serial dilution using Diluent 2. The plate was washed 3 times with prepared wash buffer (1X PBS + 0.05% Tween). Samples and standards/calibrators were run in duplicates. A volume of 50 ul of prepared samples and calibrators were added per well. The plate was sealed and incubated at room temperature for 2 hours with vigorous shaking (~ 7000 rpm). The plate was washed 3 times with wash buffer. Detection antibody was prepared by combining 60 ul of each of the SULFO-TAC Anti-human INF-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α antibodies with 2400 ul of Diluent 3. A volume of 25 ul of detection antibody solution was added to each well. The plate was sealed and incubated at room temperature for 2 hours with vigorous shaking. The plate was washed 3 times with wash buffer. Read buffer (2X) was prepared by combining 10ml of Read Buffer T (4X) with 10 ml of deionised water. A volume of 150 ul of read buffer was added to each well and the plate was then analysed on the MSD instrument. The sample concentrations (expressed in pg/ml) were directly obtained from the MSD discovery

workbench 4.0 software after 4-parameter logistic regression curves were calculated for each assay by the software.

Notes:

- All results were under 1.5 million counts to avoid overlays
- All samples were checked to see where they fit within the standard curve. For IL-13, IL-1β, IL-2 and IL-4, many samples were out of range and below the lower limit of detection (LLOD) – highlighted in grey in *Table 13*.

Table 12 shows the sensitivity characteristics of the assay. The lower limit of detection (LLOD) is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). The upper limit of quantification (ULOQ) is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% on the known value. The lower limit of quantification (LLOQ) is the lowest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% on the known value. The quantitative range of the assay lies between the LLOQ and the ULOQ and both values are kit specific and are provided in the certificate of analysis of each kit (Table 13, 14, 15).

Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again. A new standard curve was made for each plate.

<u>Table 12:</u> MSD assay specificity and sensitivity

Assay	**Samples**	**Median lower limit of detection (LLOD) (pg/ml) - kit**	**Lower limit of detection (LLOD) range (pg/ml)**	**Lower limit of detection (LLOD) (pg/ml) – assay specific - *Plate 2***	**Lower limit of quantification (LLOQ) (pg/ml) - Kit**	**Upper limit of quantification (ULOQ) (pg/ml)- Kit**
INFγ	Within range	0.20	*0.05-0.62*	0.227	7.47	938
IL-10	Within range	0.03	*0.01-0.15*	0.0369	0.680	233
IL12p70	Within range	0.11	*0.02-0.89*	0.0705	1.22	315
IL-13	Below range	0.24	*0.03-0.73*	0.508	4.21	353
IL-1β	Below range	0.04	*0.01-0.27*	0.0586	2.14	375
IL-2	Below range	0.09	*0.01-0.29*	0.0500	0.890	938
IL-4	Below range	0.02	*0.01-0.05*	0.0139	0.450	158
IL-6	Within range	0.06	*0.01-0.11*	0.0461	1.58	488
IL-8	Within range	0.04	*0.01-0.15*	0.0532	1.13	375
TNF-α	Within range	0.04	*0.01-0.13*	0.240	0.690	248

***Table 13*: LLOD and ULOD for all MSD plates**

		Plate 1		Plate2		Plate 3		Plate 4		Plate 5		Plate 6		Plate 7		Plate 8		Plate 9		Plate 10	
pg/ml	LLOD Range	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD	LLOD	ULOD
IFN-γ	*0.05-0.62*	0.244808867	1730	0.227115	1730	0.414597	1730	0.173964	1730	0.524654	1730	0.518077	1730	0.215284	1730	0.600931	1730	0.588312	1730	185.85	860.4806
IL-10	*0.01-0.15*	0.064040228	370	0.036868	370	0.021192	370	0.018854	370	0.023467	370	0.019006	370	0.050341	370	0.028973	370	0.030361	370	165.15	175.2978
IL-12p70	*0.02-0.89*	0.11146197	496	0.070479	496	0.103041	496	0.057392	496	0.051144	496	0.078394	496	0.061885	496	0.059301	496	0.053894	496	156.15	235.2256
IL-13	*0.03-0.73*	0.386130079	504	0.508292	504	0.421683	504	0.363534	504	0.295871	504	0.299856	504	0.283343	504	0.306452	504	0.531063	504	123.3	276.7011
IL-1β	*0.01-0.27*	0.135322795	566	0.058639	566	0.219547	566	0.024395	566	0.072278	566	0.049246	566	0.264571	566	0.070767	566	0.182093	566	1301.4	275.7704
IL-2	*0.01-0.29*	0.116676073	1620	0.049997	1620	0.05605	1620	0.043558	1620	0.043792	1620	0.044943	1620	0.062173	1620	0.073191	1620	0.069579	1620	322.2	774.2651
IL-4	*0.01-0.05*	0.011580485	249	0.013922	249	0.011264	249	0.010609	249	0.013834	249	0.011177	249	0.012832	249	0.011924	249	0.028824	249	128.7	123.597
IL-6	*0.01-0.11*	0.034329577	756	0.046051	756	0.04607	756	0.042457	756	0.039955	756	0.054009	756	0.050138	756	0.059345	756	0.087402	756	102.6	395.0765
IL-8	*0.01-0.15*	0.055290191	661	0.053212	661	0.0711	661	0.03995	661	0.038033	661	0.038207	661	0.046826	661	0.034993	661	0.052971	661	143.1	333.1372
TNF-α	*0.01-0.13*	0.191278756	373	0.240455	373	0.100063	373	0.08015	373	0.058482	373	0.065091	373	0.067101	373	0.053406	373	0.090883	373	172.35	190.4858

***Table 14*: Intra Assay coefficient of variation (CV) of the MSD assays**

MSD Assay	IFN-γ	IL-10	IL-12p7	IL-13	IL-1β	IL-2	IL-4	IL-6	IL-8	TNF-α
Intra Assay CV (%)	5.898262	6.92866	23.67791	31.60825	36.21415	20.9772	52.8279	4.613826	3.368413	3.158827

***Table 15:* Inter assay coefficient of variation for the MSD assays**

			Concentration (pg/ml)									
Plate	**Date**	**Internal Ctrl**	**IFN-γ**	**IL-10**	**IL-12p70**	**IL-13**	**IL-1β**	**IL-2**	**IL-4**	**IL-6**	**IL-8**	**TNF-α**
Plate 1	15/02/2017	VHR-C-008	21.541321	0.360524	0.234538	0.665355	N/A	0.274394	N/A	0.716633	13.84981	8.684077
Plate 2	16/02/2017	VHR-C-008	18.601382	0.356337	0.213141	0.665355	N/A	0.199817	N/A	0.723006	12.2595	6.915715
Plate 3	17/02/2017	VHR-C-008	15.075707	0.33638	0.240611	N/A	N/A	N/A	0.008162	0.70105	12.57177	6.607163
Plate 4	17/02/2017	VHR-C-008	12.986779	0.370859	0.157108	N/A	N/A	0.096189	N/A	0.67267	9.258658	4.607896
Plate 5	22/02/2017	VHR-C-008	14.47852	0.364889	0.236633	0.455494	N/A	0.152862	N/A	0.671077	10.06468	5.177284
Plate 6	22/02/2017	VHR-C-008	14.264836	0.323689	N/A	N/A	N/A	0.071974	N/A	0.639311	10.20554	5.265359
Plate 7	23/02/2017	VHR-C-008	16.832725	0.376562	0.128553	N/A	N/A	0.183486	N/A	0.737023	9.944691	4.799646
Plate 8	23/02/2017	VHR-C-008	14.630407	0.401266	0.199278	N/A	N/A	0.151286	0.027219	0.736956	9.164484	4.510785
Plate 9	03/03/2017	VHR-C-008	17.980485	0.510732	0.187346	0.592342	N/A	0.188192	N/A	0.849976	10.55634	4.460415
Plate 10	10/04/2017	VHR-C-008	14.378901	0.347319	0.121509	N/A	N/A	0.096801	N/A	0.666216	8.482261	3.992848
Mean concentration			16.077106	0.374856	0.190969	0.594637	N/A	0.157222	0.017691	0.711392	10.63577	5.502119
stdev of means			2.620284	0.05237	0.045946	0.098941	N/A	0.063137	0.013476	0.058828	1.712974	1.458488
CV			0.1629823	0.139706	0.240597	0.166389	N/A	0.401575	0.761727	0.082694	0.161058	0.265078
CV (%)	**Inter assay CV**		16.298231	13.97063	24.05966	16.63893	N/A	40.15749	76.17268	8.269408	16.10578	26.50776

Missing values (N/A) were below the limit of detection of the assay

2.11 Method for RNA extraction from samples stored in RNAlater® Stabilization solution using the RiboPure™ RNA Purification Kit - AM1924- Ambion (California, USA)

The protocol was followed according to the manufacturer's instructions. All centrifugation steps were carried out at 16000 RCF. Samples were all previously stored in RNAlater® Stabilization solution and maintained at -80◦C until further extraction. Samples were thawed and then centrifuged for 1 minute. The blood cells and plasma proteins formed a large brown or reddish-brown pellet which smeared sometimes upward along the side of the tube, and the supernatant was pale pink, brown, or colourless. The supernatant and all the fluid above the pellet was thoroughly removed. A volume of 800 ul of Lysing Solution and 50 ul of Sodium Acetate Solution was added to the cell pellet from RNAlater-stabilised samples. The samples were vortexed vigorously to lyse the blood cells and the tube was inverted to be sure the solution was homogenous. A volume of 250 uL of Acid-Phenol - Chloroform was added it to the cell lysate, and the samples were vortexed for 30 sec. The mixture was stored at room temperature for 5 min. The samples were then centrifuge at room temperature for 1 min to separate the aqueous and organic phases. The aqueous (upper) phase containing the RNA (1-1.2 ml) was transferred to a new tube. To each tube of aqueous phase recovered after the Acid-Phenol: Chloroform extraction, 600 uL (~one-half volume) of 100% ethanol was added and the tubes were and vortexed briefly but thoroughly. A volume of 700 ul of the mixture (aqueous phase mixed with ethanol) was added one at a time to a Filter Cartridge assembly, and the tubes were centrifuged for 30 seconds to pass the liquid through

the filter. The flow-through from the Collection Tube was discarded, and the Filter Cartridge was replaced into the same Collection Tube.

The same procedure was repeated until all the mixture was filtered. A volume of 700 uL of Wash Solution 1 was applied to the Filter Cartridge assembly and centrifuge for 30 seconds to pass the solution through the filter. The flow-through from the Collection Tube was discarded, and the Filter Cartridge was replaced into the same Collection Tube. 700 uL Wash Solution 2/3 (working solution mixed with ethanol) was applied to the Filter Cartridge assembly and centrifuged for 30 seconds to pass the solution through the filter. The flow-through from the Collection Tube was discarded, and the Filter Cartridge was replaced into the same Collection Tube. This step was repeated a second time. After discarding the flow-through from the last wash, the Filter Cartridge was replaced in the same Collection Tube and the assembly was spun for 1 min to remove residual fluid from the filter. The Filter Cartridge was transferred into a labelled Collection Tube and 70 uL of Elution Solution (preheated to ~75°C) was applied to the centre of the filter. The assembly was left at room temperature for ~20 sec, then spun for ~20–30 sec at maximum speed to recover the RNA. This step was repeated with another 70 ul of Elution Solution.

After recovering the RNA, a DNase I treatment was performed in order to remove contaminating genomic DNA from the eluted RNA. 1/20th of the volume of 20X DNase Buffer and 1 µL DNase I (8 U/uL) was added to the eluted RNA. The mixture was incubated at 37◦C for 30 minutes. A volume of DNase Inactivation Reagent equal to 20% of the volume of RNA treated was then added. The tube was vortexed briefly to thoroughly mix the DNase Inactivation reagent with the RNA, and the sample was

stored at room temperature for 2 min. The samples were then centrifuged for 1 minute to pellet the DNase Inactivation reagent. The RNA was then transferred into a new RNase-free tube. The yield and contamination ratios of the RNA were measured using the NanoDrop 2000/2000c Spectrophotometer.

Notes:

- Nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively. A ratio of ~2.0 is generally accepted as "pure" for RNA.
- Similarly, absorbance at 230 nm is accepted as being the result of other contamination (residual phenol, residual guanidine...) The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2 (Table 16)

***Table 16*: RNA yield.** *Concentrations and ratios obtained following RNA extraction of RiboPure-Blood kit®*

Sample ID	Concentration ng/ul	A(260)/A(280)	A(260)/A(230)
VHR-C-043	48.3	2.09	1.95
VHR-C-044	95.6	2.09	2.02
VHR-C-045	3.4	5.5	0.38
VHR-C-046	58.5	2.17	1.91
VHR-C-047	62	2.14	2.02
VHR-C-048	59.7	2.19	1.94
VHR-C-049	58.1	2.16	2.04
VHR-C-050	63.2	2.14	1.9
VHR-C-051	19	2.46	1.71
VHR-C-052	98.1	2.12	1.9
VHR-C-053	78	2.1	1.86
VHR-C-054	41.5	2.27	1.73
VHR-C-055	35.8	2.22	1.58
VHR-C-056	54.7	2.15	1.9
VHR-C-057	86.4	2.17	2.09
VHR-C-058	70.3	2.14	2.04

VHR-C-059	57.3	2.15	1.72
VHR-C-060	92.3	2.13	1.85
VHR-C-061	53.8	2.12	1.45

2.12 Method for making up cDNA using the Transcriptor First Strand cDNA Synthesis Kit – 04379012001 – Roche (Mannheim, Germany)

Note: Suitable template concentrations may range from 10ng to 5 ug total RNA and from 1 to 100 ng of mRNA

A total of 300 ng of RNA of each extracted RNA sample was converted to cDNA.

For each tube, the appropriate volume of RNA corresponding to 300 ng was mixed with PCR grade water (vial 7 - when needed) to make the volume up to 11 ul and then 2 ul of Random Hexamer Primer (600 pmol/ul -vial 6) was added to each tube. The template-primer mixture was denatured by heating the tube for 10 minutes at 65◦C in a thermal block cycler (Techne – Prime – Bibby Scientific) with a heated lid to minimise evaporation. This steps ensures the denaturation of RNA secondary structures. The tubes were then immediately cooled on ice. To each tube 4 ul of Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc (vial 2), 0.5 ul pf Protector RNase Inhibitor, 40U/ul (vial 3), 2 ul of Deoxynucleotide Mix, 10 mM each (vial 4) and 0.5 ul Transcriptor Reverse Transcriptase 20U/ul (vial 1) were added. The tubes were then centrifuged briefly and placed in a thermal block cycler with a preheated lid. The Reverse Transcriptase reaction was incubated for 10 minutes at 25◦C, followed by 60 minutes at 50◦C. The Transcriptor Reverse Transcriptase was then inactivated by heating the tubes to 85◦C for 5 minutes and then the temperature

was set to 4◦C to cool the tubes down. The tubes were then stored at -20◦C until further applications.

Controls:

For each batch of converted RNA:

- A water control was used to control the PCR grade water used to make up the RNA. In this tube, PCR grade water was added instead of the RNA.
- A Transcriptor Reverse Transcriptase (minus RT) control was used to control for any contamination of the reagents with the enzyme. In this tube, the enzyme was not added to the tube.
- A technical control was prepared to run on each real-time PCR plate in order to monitor plate to plate variation.

These controls for the cDNA step were then used for the quantitative PCR.

2.13 Method for real-time polymerase chain reaction using Roche Light cycler®480 II and the LightCycler® 480 probe Master reagents (04707494001) and probes – Roche (Mannheim, Germany)

A total volume of 10 ul of PCR mix was prepared for each well of the Multiwell Plate and each sample was run in triplicate. GAPDH was chosen as the reference gene. A volume of 1 ul of sample cDNA was mixed with 1.5 ul of PCR grade water to make up a total of 2.5 ul of cDNA template for each well. A PCR mix was prepared by combining 2 ul of PCR grade water (vial 2), 5 ul of LightCycler® 480 Probes Master 2x conc (vial 1) and 0.5 of either GAPDH (Assay ID 141139), ADAM17/TACE (Assay ID 136022), NLRP3 (Assay ID 102938), TIMP3 (Assay ID ID 101221) or IL1B (Assay ID100950)

RealTime ready assay probes in each well. The plate was then sealed with the LightCycler® 480 Sealing Foil and centrifuged for 2 minutes at 1500 RCF. The plate was then transferred into the plate holder of the Roche Light cycler®480 II Instrument and the run program was started. The run program 'Monocolor Hydrolysis Probe/UPL Probe' was selected among the experiment template offered by the LightCycler® 480 Software Release 1.5.1.62 software which is a 45-cycle amplification and the volume was changed to 10 ul. The run was started and the experiment was saved.

Controls:

On each plate:

- The PCR grade water and the minus RT control prepared from the cDNA step were run.
- The same technical control was run on each real-time PCR plate
- A probe control was run. This was a probe and probe master mix control mixed with PCR grade water instead of the template cDNA
- A cDNA control was run. This was a cDNA template mixed with PCR grade water instead of the probe and probe master mix.

Results analysis:

Samples, target and reference genes were labelled in the 'Sample editor' menu (following the plate plan) after choosing the 'relative quantification' option in the LightCycler® 480 Software Release 1.5.1.62 software. cT (Cycle Threshold) values were then calculated in the 'Analysis' menu after selecting 'Abs Quant/2nd Derivative

Max' option. The table was then exported to excel and the mean as well as the standard deviation were calculated for each triplicate.

Note: The Ct (cycle threshold) value is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level).

In order to normalise the expression of our target gene (TACE in this example) to the reference gene, ΔcT values were calculated by subtracting the cT value of the reference gene from the cT value of the target gene:

ΔcT (TACE) = cT (TACE) – cT (GAPDH)

2^(-ΔcT) was calculated for each sample which represents the fold increase in gene expression of the target gene compared to the reference gene.

The 2^(-ΔcT) values were then compared among the studied groups.

Gene efficiencies calculations

Gene efficiencies calculations were performed prior to real-time PCR relative quantification step. This was done by using a serial dilution of a template cDNA and then quantifying the gene of interest as well as the reference gene in our serial dilution set of samples. Each gene efficiency was then calculated using a standard curve using the LightCycler® 480 SW 1.5.1 software and selecting absolute quantification as the analysis method.

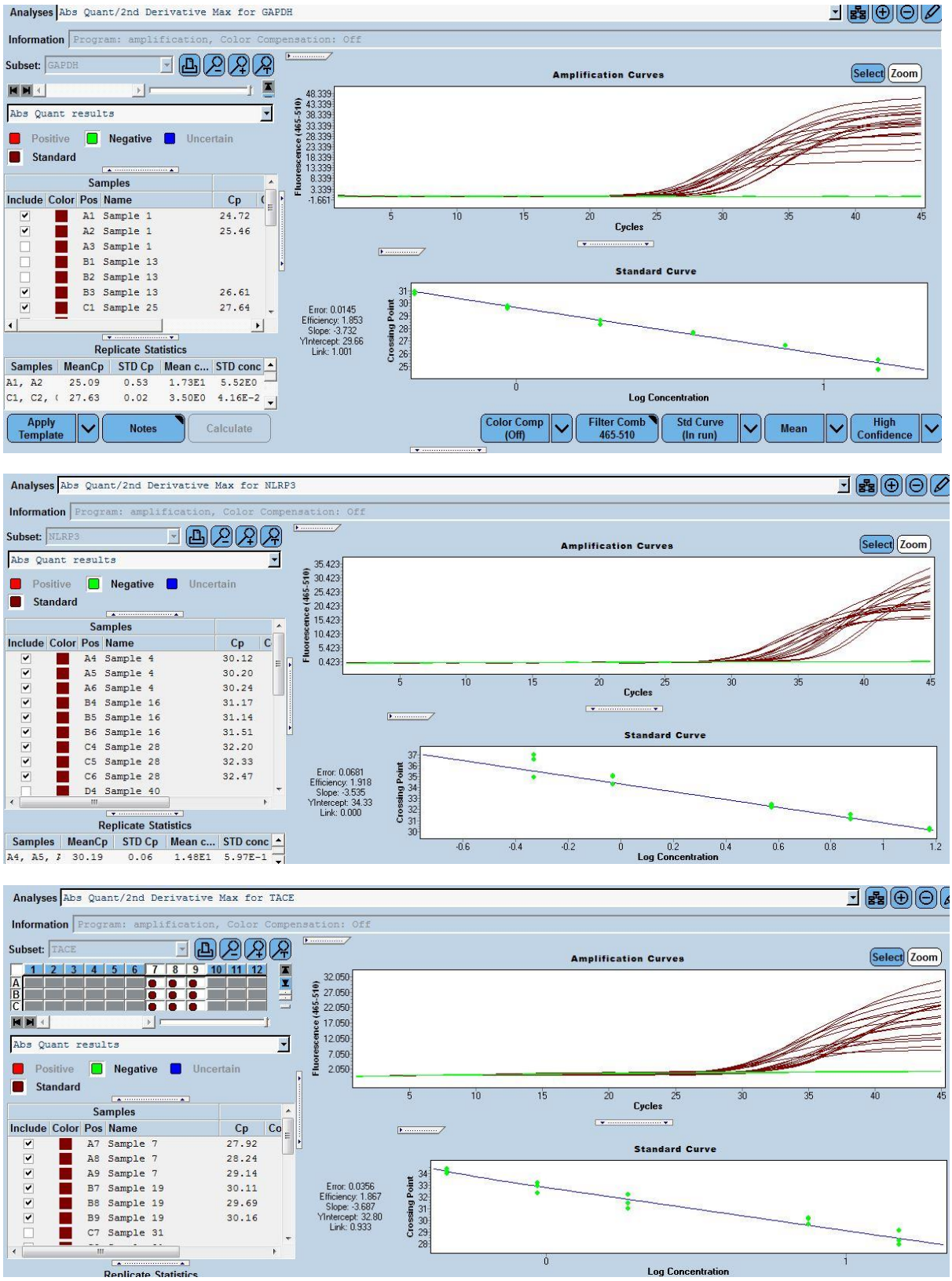


Figure 7: ***Gene efficiencies of TACE, NLRP3 and GAPDH***

Only a coefficient of variation (CV) of less than 20% was accepted between replicates. If one replicate was out of this range, the remaining duplicates were used for the analysis. Samples with more than one replicate out of range were analysed again.

Table 17: ***Inter-assay coefficient of variation (CV) of the Cp values for*** **GAPDH, NLRP3** ***and*** **TACE** ***genes***

Inter assay CV (%) for all real-time PCR plates			
6 plates			
VHR-217 (Low Cp value)	***GAPDH***	***NLRP3***	***TACE***
Average	22.81667	28.15389	26.28333
stdev of means	0.609338	0.587815	0.326694
CV	0.026706	0.020879	0.01243
CV (%)	2.670585	2.087864	1.24297
28 plates			
MD-009 (Medium Cp value)	***GAPDH***	***NLRP3***	***TACE***
Average	23.54238	28.53768	28.60988
stdev of means	1.107169	2.055639	1.647659
CV	0.047029	0.072032	0.057591
CV (%)	4.702874	7.203247	5.759056
8 plates			
LR-66 (High Cp value)	***GAPDH***	***NLRP3***	***TACE***
Average	28.55875	34.10333	32.08708
stdev of means	0.658611	0.718263	0.540184
CV	0.023062	0.021061	0.016835
CV (%)	2.306161	2.106137	1.683492
Total Inter-assay CV (%)			
Average of high, medium and low technical controls			
Gene	***GAPDH***	***NLRP3***	***TACE***
Inter-assay CV (%)	3.22654	3.799083	2.895173

Table 18: ***Inter-assay Coefficient of variation for the Cp values of*** **GAPDH, TIMP3** ***and*** **IL1B** ***genes***

		GAPDH	TIMP3	IL1B
CTRL	Plate 1	27.55	N/A	29.97333
CTRL	Plate 2	27.27333	37.55	29.49667
CTRL	Plate 3	26.87333	37.99	29.64667
CTRL	Plate 4	27.06333	38.17	29.37667
CTRL	Plate 5	27.34	38.41	29.68
CTRL	Plate 6	27.18333	37.47	30.05667
CTRL	Plate 7	27.62	39.03	30.87333
CTRL	Plate 8	27.13667	38.015	30.30667
CTRL	Plate 9	26.81	37.82	29.82667
Inter- assay CV				
Average		27.20556	38.05688	29.91519
Std dev of means		0.275086	0.499735	0.460314
CV		0.010111	0.013131	0.015387
CV (%)		**1.011138**	**1.313127**	**1.538731**

2.14 Method for analysing samples using the Proximity Extension Assay (PEA) technology - Proseek® Multiplex provided by O-Link Proteomics (Uppsala, Sweden)

O-Link proteomics performs a detection and sample analysis by high-throughput real-time PCR analysis using the Fluidigm® BioMark™ HD System.

The main steps involved in a Proseek assay are outlined below and this analysis was undertaken and provided by O-Link proteomics®. A pair of oligonucleotide-labeled antibodies, known as Proseek probes, bind to the target protein present in the sample. When the two Proseek probes are in close proximity, a new PCR target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time PCR. Each of the 96-oligonucleotide antibody-pairs contains unique DNA sequences allowing hybridization only to each other. Subsequent proximity extension creates 96 unique DNA reporter sequences which are amplified by real-time PCR. A limiting

factor of multiplexed immunoassays is the antibody cross-reactivity which restricts the degree of multiplexing of most assays to below 10-plex. Cross-reactive events are not detected with Proseek Multiplex since only matched DNA reporter pairs are amplified with real-time PCR. This allows for scalable multiplexing without loss of specificity and sensitivity. http://www.olink.com/data-you-can-trust/technology/

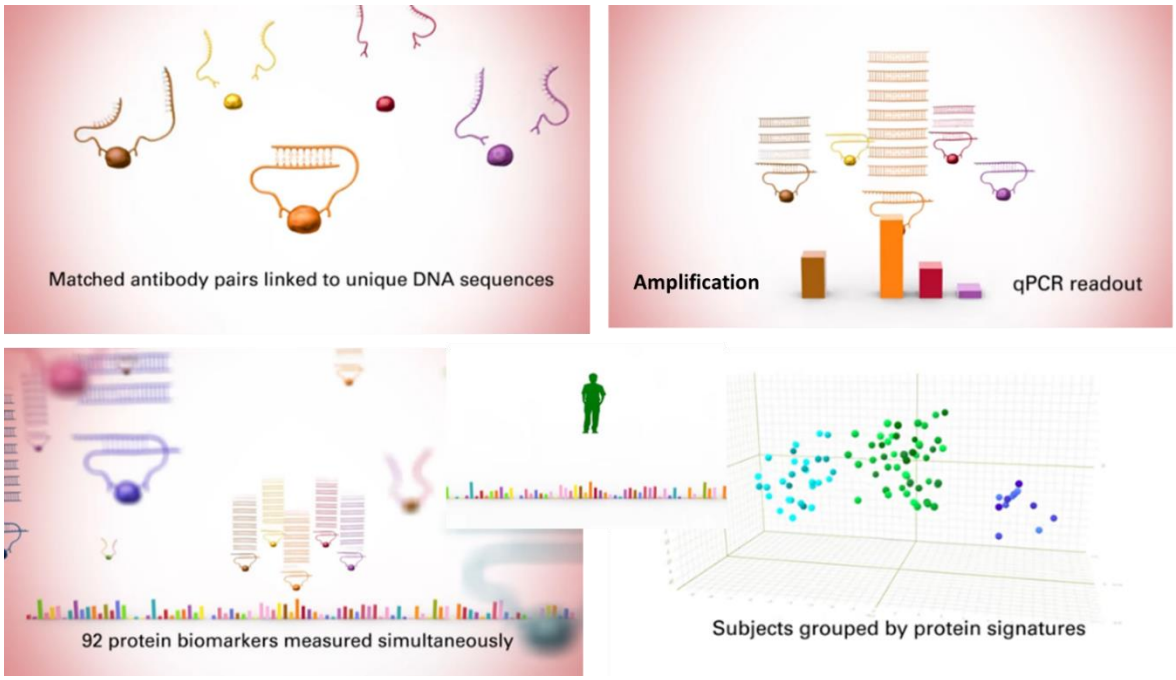


Figure 8: ***Proximity Extension Assay (PEA) technology steps***

In order to prepare the samples, a sample map was created for all the samples that would be analysed and 30 ul of plasma was pipetted into each well of a 96-well plate. A total of 4 plates were prepared. The plates were then sealed, covered, placed on ice and shipped to O-Link Proteomics lab in Uppsala, Sweden for protein measurement. The assay results were sent back and the statistical data analsysis was carried out as part of this project and results are in **Chapter 6**.

Controls:

Internal controls: added to each individual sample

- 2 incubation controls: Non-human antigens which monitor potential variation in all three steps.
- 1 extension control: An antibody with both DNA-tags always in proximity which monitors the extension step and is used for normalisation across the samples
- 1 detection control: A complete double stranded amplicon which controls the Amplification/Detection steps

External controls: Added to separate wells on the plate

- Inter-plate control (IPC): Pool of 92 antibodies, each with one of the pairs of unique DNA tags on it positioned in fixed proximity (i.e 92x extension control). Used for normalisation and compensates for potential sequence biases and variation between runs/plates.
- Negative control: Buffer with no antigens. Sets the background levels (LOD) for all proteins.

The quality of the entire run is evaluated by calculating the standard deviations of incubation control 1, incubation control 2 and detection control. These values should be below a pre-determined quality threshold. The quality of each sample is assessed by evaluation of the deviation from the median value for incubation control 2 and detection control. The recommended maximum deviation is ± 0.3. The IPC controls are used for studies including more than 90 samples and there is a third quality control that compares the LOD values against a predetermined interval.

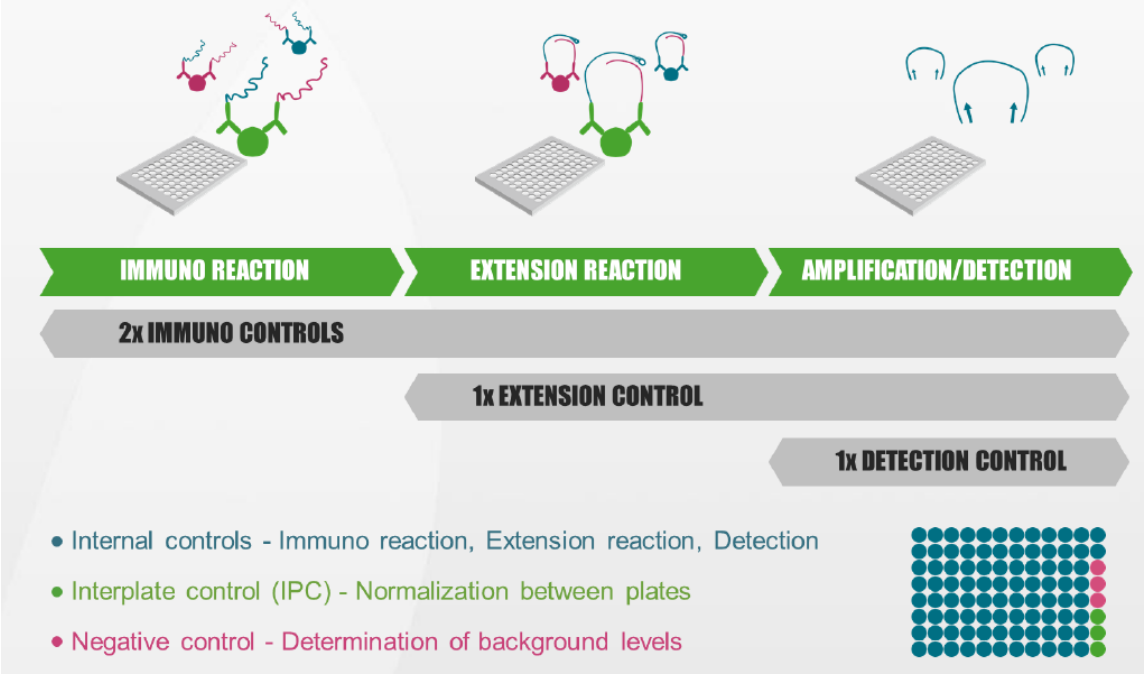


***Figure 9:* Controls used for the Proseek Multiplex technique**

Results Analysis:

Proseek Multiplex uses relative quantification. The data from the analysis is presented as normalized protein expression (NPX) values, which is an arbitrary unit on log2 scale. This means that an increase in one NPX corresponds to a doubling of the concentration. Proseek is a relative quantification method which means that even if two proteins have the same NPX values, their actual concentration may differ. NPX is generated by a combination of the Fluidigm multiplex real-time PCR system and O-Link's data normalisation procedure and is performed to minimise both intra- and inter-assay variation. Samples where values were below the detected level were replaced with 'Not a number/NaN'.

Data pre-processing and analysis – stepwise overview: Proseek generates Cq values and NPX values are obtained by the following method: *(Normalization and quality control by Olink Wizard with GenEx software)*

1.Extension Control

dC qanalyte = Cq analyte – Cq Ext Ctrl

2.Interplate Control

ddCq analyte = dCq analyte – dCq Interplate Ctrl

3.Normalization against a correction factor

NPX = Correction factor – dd Cqanalyte

Normalized Protein eXpression (NPX) values

Log 2 scale: high NPX value = high protein concentration

For CV calculations, linear 2^{NPX} values were used

Linear values: high linear value = high protein concentration

5.

INTRA AND INTER CV (average this data set)

CV is calculated using linear values (2^{NPX}).

	CVD II (average)	CVD III (average)
Intra CV	4%	5%
Inter CV	9%	10%

INTRA CV (No. of assays)

CV (%)	CVD II	CVD III
<5	75	64
5-10	15	25
10-15	2	2
15>	0	1

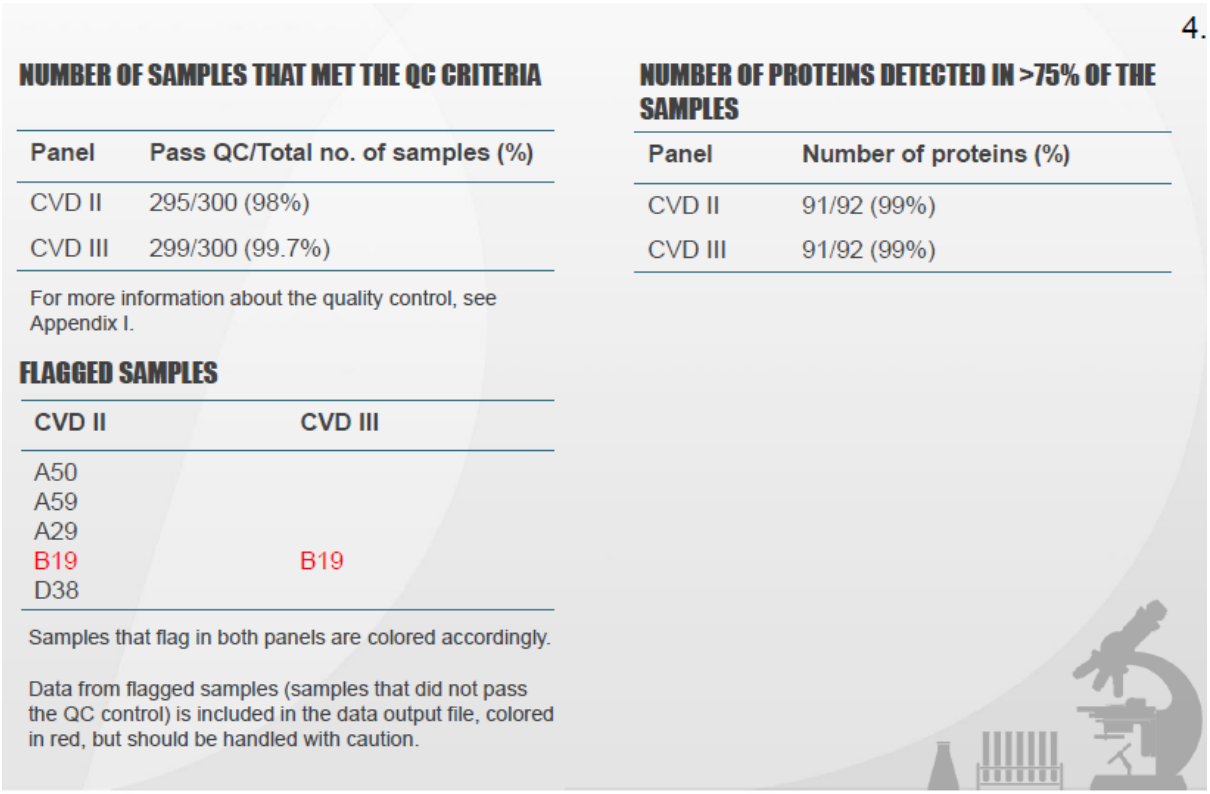
INTER CV (No. of assays)

CV (%)	CVD II	CVD III
<10	76	65
10-20	15	24
20-30	0	2
30>	1	1

Inter and intra CV

OLINK PROTEOMICS

Figure 10: ***Inter and intra-assay coefficient of variation of the proteins analysed by O-link for cardiovascular disease panel II (CVDII) and III (CVDIII).***



4.

NUMBER OF SAMPLES THAT MET THE QC CRITERIA

Panel	Pass QC/Total no. of samples (%)
CVD II	295/300 (98%)
CVD III	299/300 (99.7%)

For more information about the quality control, see Appendix I.

FLAGGED SAMPLES

CVD II	CVD III
A50	
A59	
A29	
B19	B19
D38	

Samples that flag in both panels are colored accordingly.

Data from flagged samples (samples that did not pass the QC control) is included in the data output file, colored in red, but should be handled with caution.

NUMBER OF PROTEINS DETECTED IN >75% OF THE SAMPLES

Panel	Number of proteins (%)
CVD II	91/92 (99%)
CVD III	91/92 (99%)

Quality control

OLINK PROTEOMICS

Figure 11: ***Quality control measurements of the proteins analysed by O-link for cardiovascular disease panel II (CVDII) and III (CVDIII).***

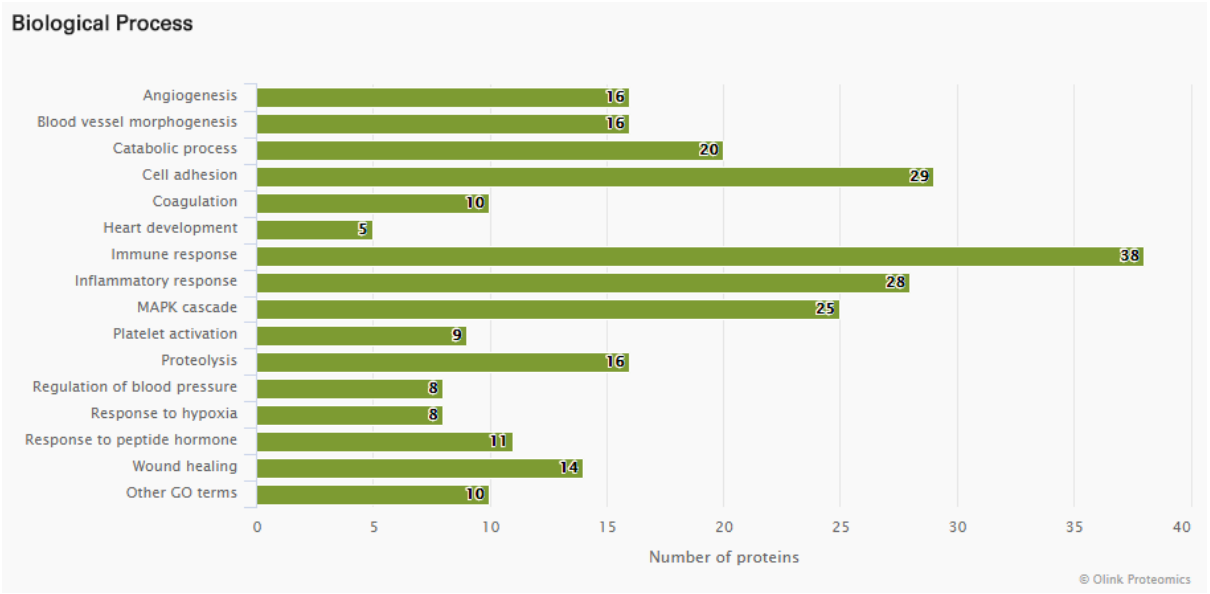


Figure 12: ***Cardiovascular disease panel II (CVDII).*** *Biological processes from Genontology*

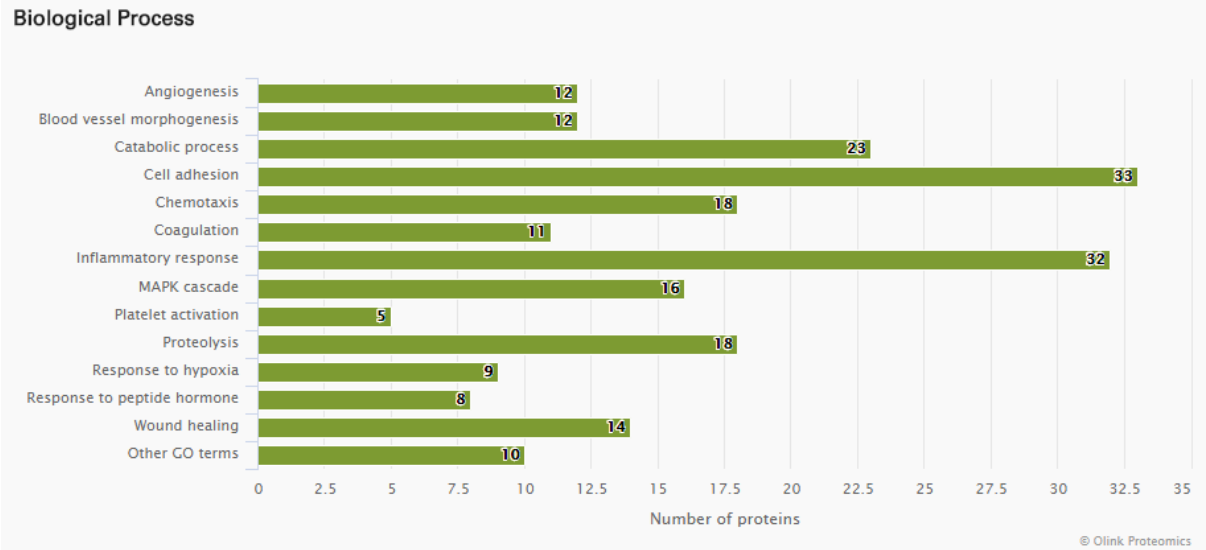


Figure 13: ***Cardiovascular disease panel III (CVDIII).*** *Biological processes from Genontology*

2.15 Methods for performing the colony forming assay (CFA)

The protocol for performing the CFA was adapted form Martin-Ramirez et al ((160) and Ingram et al (161) – with minor modifications.

2.15.1 Preparation of collagen coated 96-well plates

A collagen type I solution was prepared (Corning® collagen I, eat tail, 354236, Maryland, USA). The collagen solution was diluted to 50 ug/ml using a 0.02N acetic acid solution (where the acid was previously filtered using a 0.2 um filter) as the Corning Collagen I is insoluble at neutral pH. A plate of 96-wells was coated using the diluted collagen type I solution at 5 ug/cm^2 with 50 ul of collagen solution per well. The plate was incubated at room temperature for one hour before the liquid was aspirated and the plate washed three times with PBS.

2.15.2 Preparation of endothelial colony forming cells (ECFCs) culture medium

The reagents enclosed in the EGM-2 BulletKit (Lonza - CC-3162, Maryland, USA) were thawed. hEGF, GA-100 (gentamycine, amphotericin-B), FBS, VEGF (vascular endothelial growth factor), hFGF-B (human fibroblast growth factor), R3-IGF-1 (insulin like growth factor), ascorbic acid and heparin were added to 500 ml of EGM-2. The culture medium was also supplemented with 100 ml of additional FBS previously inactivated at 56◦C for 30 minutes. The media was aliquoted into 50 ml aliquots and some were frozen at -20◦C.

2.15.3 White blood cell isolation

A total of 18 ml of blood was collected into EDTA vacunatiner blood tubes (VACUETTE K3E K3EDTA – 455036). The blood tubes were kept at room temperature until processing. Isolation of peripheral mononuclear blood cells (PBMCs) was then performed using the Histopaque®-1077 from sigma (10771). The volume of 18 ml of blood was diluted 1:1 with PBS and then for every 9 ml of diluted blood, 4.5 ml of Histopaque-1077 was used. The 4.5 ml of Histopaque-1077 were firstly added at the bottom of the tube and the diluted blood was slowly and gently layered on top of the Histopaque-1077 layer while making sure that the ficoll layer is not disturbed.

The tubes were then centrifuged at 2000 rpm for 20 minutes. The layer of white blood cells was then collected into a new fresh tube. The cells were then washed twice with PBS by spinning at 1500 rpm for 15 minutes. The cell pellet was then gently suspended in 15 ml BOEC medium and then aliquoted at 150 ul of cell suspension per well. The cells were incubated at 37◦C 5%CO2 for 24 hours and then the non-adherent cells were discarded. The media was replaced every two days the first week and every three days the following weeks. In general, colonies take around three weeks to appear. After three weeks, the number of colonies were counted. Cells were identified as well-circumscribed monolayers with a cobblestone morphology as shown in the picture below.

***Figure 14**: ECFC isolated from patient VHR-030 after CD45 depletion by autoMACS* *(50 x magnification)*

Chapter 3

TACE/ADAM17 and TNFα identify and further stratify individuals at very high risk of cardiovascular events

Abstract

Background: Tumour necrosis factor alpha converting enzyme (TACE) is known to cleave tumour necrosis factor alpha (TNFα) amongst many other cytokines and inflammatory mediators associated with increasing the risk of major adverse cardiovascular events (MACE). *TACE* gene expression was found to be increased in acute myocardial infarction and complications such as heart failure and arrhythmias. However, TACE plasma protein levels have not yet been explored in CVD individuals. The aim of this study was to measure TNFα and TACE in individuals at various levels of cardiovascular risk.

Methods: Participants were recruited from the cardiac catheterisation laboratory or by email advertisement. All participants were assigned a risk score using the European Society of Cardiology (ESC) SCORE risk chart. Group 1 was defined as very high risk participants (VHR) with a 10-year risk SCORE ≥10% risk of fatal cardiovascular disease (CVD). Group 2 were defined as low, moderate and high risk participants (non-VHR) with a 10-year risk SCORE <10% risk of fatal CVD. TNFα plasma protein levels as well as TACE plasma and cell membrane bound protein levels were measured by ELISA. *TACE* mRNA levels were measured by quantitative real-time PCR.

Results: A total of 344 participants were recruited. TNFα plasma protein levels and *TACE* mRNA levels were significantly higher in the VHR group (n=229) compared to non-VHR group (n=115) (4.711 ± 2.453 pg/ml *vs.* 2.770 ± 0.752 pg/ml, $p<0.0001$ and 0.05366 ± 0.03127 *vs.* 0.03755 ± 0.02051, $p<0.01$ respectively). TNFα plasma protein levels remained higher in the VHR group with previous myocardial infarction (MI) (5.399 ± 2.368 pg/ml *vs.* 4.389 ± 2.433 pg/ml; $p<0.01$) or previous percutaneous coronary intervention (PCI) (5.260 ± 2.337 pg/ml *vs.* 4.454 ± 2.471 pg/ml; $p<0.01$)

despite medical and clinical management. However, *TACE* mRNA levels were significantly lower in the VHR group with a previous PCI (0.04624 ± 0.02494 *vs.* 0.05708 ± 0.03331 with $p<0.05$). There was no significant difference in TACE plasma protein levels between VHR and non-VHR groups ($p=0.642$). The VHR group however, had significantly higher cell membrane bound TACE levels compared to the non-VHR group (383.9 ± 259.6 pg/ml in VHR *vs.* 221.9 ± 78.8 pg/ml in non-VHR participants, $p<0.0001$).

Conclusion: Results demonstrate that TNFα and TACE can identify VHR individuals and may add value to current risk prediction models. Additionally, this study shows that despite clinical management in VHR participants with a previous PCI, TNFα plasma levels remain high whereas *TACE* mRNA levels are low. This suggests that there are mechanisms in place behind *TACE* downregulation and TNFα over secretion after a PCI that need further understanding. On the other hand, this is the first study to measure both *TACE* gene expression and TACE protein levels (plasma and cell bound form) in individuals at various levels of cardiovascular risk. This study also provides preliminary evidence for the cell membrane bound form of TACE as valuable target for future therapy development. Additional studies are however required to investigate this and the utility of the TNFα family as biomarkers for predicting MACE risk.

3.1 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Coronary Artery Disease (CAD), the largest contributor of CVD, accounts for 6.7 million deaths worldwide (WHO global status report on noncommunicable diseases, 2014). Importantly, most patients who die from CAD have no previous symptoms and currently, there is no definitive way to predict cardiovascular events. Clinicians rely on the use of risk factors such as age, high cholesterol, obesity, smoking, high blood pressure, diabetes, and chronic kidney disease, some of which are used in scoring systems, to predict who is most susceptible. However, these scoring systems have been intensely criticised because of their lack of predictive values in all cases as some patients who are assigned a low score based on one risk factor develop CAD while others who are assigned high scores never develop CAD. There is therefore a critical need to develop better tests to predict those at risk of either primary or secondary cardiovascular events.

CAD initiation and progression is due to atherosclerosis which is the result of an imbalanced lipid metabolism and a maladaptive immune response leading to chronic inflammation in the arterial wall (6,7). Atherosclerosis was once thought to be associated with a passive build-up of plaque but is now recognised as an inflammatory disease (162). Some inflammatory pathways are known to have involvement in the initiation of the atherosclerotic lesion (163) while others have been shown to play an important role in plaque rupture and thrombosis (164). Measuring a combination of inflammatory markers specific to each pathway may

uncover the underlying state of the disease and the likelihood of the occurrence of major adverse cardiovascular events (MACE).

In the present study, the inflammatory pathway involving tumour necrosis factor alpha (TNFα) and tumour necrosis factor alpha converting enzyme (TACE) (also known as ADAM17) is explored. TACE is a membrane bound protein responsible for the ectodomain shedding of a variety of inflammatory markers, including TNFα, most of which play role in the initiation and progression of CAD (151,152). It is already well-established that TNFα levels are higher in participants with an acute myocardial infarction (AMI) (153,154) with higher levels in AMI participants compared to stable angina participants (165). Since TNFα has been extensively investigated in CAD, studying the main enzyme responsible for cleaving membrane TNFα could provide further insight on the role of this pathway in CAD. *TACE* gene expression levels and surface protein expression have been measured in previous studies either by quantitative real-time PCR, immunohistochemistry or flow cytometry techniques. In those studies, TACE was found to be increased in myocarditis (166), advanced congestive heart failure (167), aortic aneurysm (168), hypertension (169), AMI (165), AMI and complications such as heart failure and arrhythmias (170,171) and atherosclerotic ischemic stroke (172). However, despite the major role of TACE in several inflammatory processes, the assessment of its value as a potential CVD biomarker by measuring its plasma levels in CVD individuals hasn't been previously explored. TACE internalisation is its major form of its self-elimination (173,174). However, TACE has been detected on the surface of microparticles released from platelets and endothelial cells in the plasma (175,176). To date, TACE plasma levels have been measured only in few studies (active proteinase-3 (PR3)-positive ANCA-

associated vasculitis (AAV), clinical malaria and Alzheimer's disease (176–178), but were not previously measured in individuals with CAD. The main aim of this study was therefore to measure the different forms of TACE protein in terms of plasma, cell membrane bound and gene expression levels together with TNFα plasma levels in order to assess their role in CVD risk stratification.

3.2 Participants and Methods

Refer to **section 2.1** in **Chapter 2** for a detailed description of the participant recruitment process, the risk score used and the methods for blood processing.

3.2.1 Demographic information

The presence of comorbidities such as hypertension, diabetes, chronic kidney disease (glomerular filtration rate (GFR) < 60 ml/min), arthritis, depression or any other condition was recorded for each participant after consulting the participant's hospital notes as well as the Northern Ireland electronic care record (NIECR). The presence of a previous myocardial infarction (MI), percutaneous coronary intervention (PCI), Coronary artery bypass graft (CABG) or stroke were recorded. Hyperlipidaemia was considered present if 1 of 3 criteria was met: total cholesterol ≥5.0 mmol/L, self-reported hyperlipidaemia or if hyperlipidaemia was mentioned in the participant's notes or on the electronic care records. Medical and family history were collected from each participant. Body mass index (BMI) was calculated from baseline investigation measurements of height (cm) and weight (kg). Physical activity was ascertained from questionnaire data on leisure-time exercise during the past year. Participants who reported in the questionnaire that they smoked regularly were

classified as current smokers. Participants' employment status, alcohol consumption and diet was also recorded. No information about race/ethnicity was collected. Acute coronary syndrome VHR (ACS-VHR) participants admitted for major adverse cardiovascular events (MACE) were defined as VHR participants admitted with an ACS (unstable angina, non-ST-segment elevation myocardial infarction or ST-segment elevation myocardial infarction) who have previously had an MI, a PCI, a CABG or a stroke. Follow up MACE were defined either within 6 months or after 6 months as any deaths, admission for ACS or stroke and transient ischemic attack (TIA). The angiogram outcome was reported in terms of the severity of the atherosclerotic lesion based on the angiogram report that was issued by the consultant cardiologist. The angiogram outcome was classified as mild, moderate, severe, very severe disease, major obstruction or triple vessel disease.

3.2.2 Measurement of TNFα plasma protein levels

Refer to **section 2.10 in Chapter 2.**

3.2.4 Measurement of *TACE* gene expression

Refer to **section 2.11, 2.12 and 2.13 in Chapter 2.**

3.2.5 Measurement of plasma TACE plasma protein levels

Refer to **section 2.6.1 in Chapter 2.**

3.2.6 Measurement of cell membrane bound TACE protein levels

Refer to **section 2.4, 2.5 and 2.6.2 in Chapter 2.**

3.2.7 Statistical methods

Data analysis was performed using the IBM® SPSS version 23 software (SPSS Inc., Chicago, IL). In the analysis, cases with missing data were excluded pairwise. Normality tests were conducted for all explored continuous variables. Variables that did not follow normal distribution were transformed using a logarithmic function to allow their use in parametric methods of analysis. Descriptive statistics were carried out with standard methods using Student T-test when comparing the means of two groups, ANOVA when comparing the means of more than two groups and Pearson Chi Square test when comparing categorical variables. Continuous variables were expressed as mean ± SD whereas categorical variables were expressed as percentages. An analysis of covariates (ANCOVA - also known as a hierarchical multiple regression) was used to compare the levels of each variable between different groups while controlling for confounding factors. Because our cohorts were not age and sex matched, age and gender were controlled for in the statistical analysis. Age and gender are recognised as confounding effects because increased age as well as male gender are associated with CVD (179) and they have also shown to be associated with the variability of the measured biomarker levels in a linear regression model applied in the study (180). Within the comparisons among the VHR cohort, no adjustments were made unless a covariate was associated with both the biomarker and the outcome as previously descried paper by Kamangar *et al.* regarding confounding variables in epidemiological studies (180). Pearson's correlation was used to evaluate the correlation between variables. A logistic regression was used to examine whether the measured markers can effectively

predict the cohort membership (VHR or non-VHR). A multinomial logistic regression analysis was performed to investigate whether the measured variables can effectively predict the severity of the plaque burden. Statistical significance was defined as values of $p<0.05$ (two-tailed).

3.3. Results

3.3.1 Population Demographics

A total of 344 participants were recruited consecutively over a period of two years. The VHR (n=229) group consisted of ACS-VHR (n=127) and ELEC-VHR (n=102) participants. The non-VHR (n=115) group consisted of low risk (LR) (n=81), moderate risk (MR) (n=32) and high risk (HR) (n=2) participants. All recruited participants were assigned a risk SCORE according to the European Society of Cardiology (ESC) guidelines. The VHR group had a SCORE ≥ 10% and the non-VHR group had a SCORE < 10%. Table 1 and 2 provide the full descriptive statistics of the population. The mean age of the VHR participants at study entry was 65 years and 21% were women. A proportion of 56% had a history of hypertension, 19% were diabetic and 19% were current smokers. The mean age of the non-VHR participants was 46 years and 70% were women. A proportion of 13% percent had hypertension (who were more likely to be MR n=32) and 4 % were current smokers.

<u>Table 1:</u> Population demographics of participants at various levels of cardiovascular risk classified according to the SCORE risk chart.

	VHR	MR	LR	*p-value (One way ANOVA)*	VHR	Non-VHR	*p-value (t-test)*
Number of participants	229	32	81		229	115	
Age (years)	65	56	41	<0.0001	65	46	<0.0001
Male (n; %)	170; 78.6	16; 50.0	16; 19.7	<0.0001 (Chi 2)	170; 78.6	34; 29.6	<0.0001 (Chi 2)
Female (n; %)	49; 21.4	16; 50.0	65; 80.2		49; 21.4	81; 70.4	
Weight (Kg)	86.1 ± 19.5	81.6 ± 18.2	74.0 ± 14.7	<0.0001	86.1 ± 19.5	76.7 ± 16.6	<0.0001
Height (cm)	171.0 ± 9.5	167.8 ±10.4	166.8 ±8.8	0.0017	171 ± 9.5	167.2 ±9.3	0.0005
BMI (Kg/m^2)	29.1 ± 6.4	29.0 ± 6.2	26.7 ± 5.5	0.0087	29.1 ± 6.4	27.5 ± 5.9	0.0188
Systolic BP (mmHg)	131 ± 24	137 ± 2	119 ± 14	<0.0001	131 ± 24	124 ± 20	0.0111
Diastolic BP (mmHg)	72 ± 12	81 ± 16	74 ± 12	0.0025	72 ± 12	76 ± 14	0.0142
Total Cholesterol (mmol/L)	4.2 ± 1.3	5.0 ± 1.2	5.0 ± 0.9	<0.0001	4.2 ± 1.3	5.0 ± 1.0	<0.0001
LDL Cholesterol (mmol/L)	2.3 ± 1.0	2.9 ± 1.1	2.9 ± 0.9	<0.0001	2.3 ± 1.0	2.9 ± 0.9	<0.0001
HDL Cholesterol (mmol/L)	1.2 ± 0.5	1.6 ± 0.4	1.6 ± 0.4	<0.0001	1.2 ± 0.5	1.6 ± 0.4	<0.0001
TG (mmol/L)	1.8 ± 2.1	1.1 ± 0.5	1.0 ± 0.7	0.0019	1.8 ± 2.1	1.1 ± 0.6	<0.0001
CRP (mg/L)	12.0 ± 31.2	2.9 ± 2.3	2.4 ± 3.8	0.008	12.0 ± 31.2	2.5 ± 3.4	0.002

One way ANOVA was used to compare the VHR, MR and LR cohorts. Student t-test was used to compare VHR to non-VHR. (BMI: body mass index; BP: blood pressure; CRP: C-reactive protein; HDL: high density lipoprotein; LDL: low density lipoprotein; LR: low risk; MR: moderate risk; SCORE: Systematic COronary Risk Evaluation risk score; VHR: very high risk; non-VHR: non-very high risk; TG: triglycerides).

<u>Table 2:</u> Medical history and lifestyle characteristics of participants at various levels cardiovascular risk classified according to the SCORE risk chart.

	VHR	MR	LR	*p-value (Chi 2)*	VHR	Non-VHR	*p-value (Chi 2)*
Number of participants	229	32	81		229	115	
	n; %				***n; %***		
Hypertension	128; 55.9	9; 28.1	5; 6.17	<0.0001	128; 55.9	15; 13.0	<0.0001
Diabetes	44; 19.2	0; 0	0; 0	N/A	44; 19.2	0; 0	N/A
Dyslipidaemia	134; 58.5	20; 62.5	35; 43.2	0.0404	134; 58.5	57; 49.6	0.115
GFR <60 ml/min	45; 20.0	1; 3.4	2; 2.5	0.000154	45; 20.0	3; 2.7	<0.0001
Arthritis	35; 15.3	5; 15.6	1; 1.2	0.0029	35; 15.3	6; 5.2	0.00656
Depression	43; 18.8	4; 12.5	7; 8.6	0.0858	43; 18.8	11; 9.6	0.0267
Employed	33.3	26; 79.3	80; 98.8	<0.0001	33.3	104; 92.9	<0.0001
Current smokers	42; 19.3	3; 9.7	1; 1.2	0.00025	42; 19.3	5; 4.4	0.00022
Ex-smokers	110; 50.4	9; 29.0	35; 43.2	<0.0001	110; 50.4	45; 39.1	<0.0001

Pearson's chi square test was used to compare VHR, MR and LR groups and the VHR and non-VHR groups. (GFR: Glomerular filtration rate; LR: low risk; MR: moderate risk; non-VHR: non-very high risk; SCORE: Systematic COronary Risk Evaluation risk score; VHR: very high risk).

***Table 3:* TNFα plasma and TACE plasma and gene expression levels in participants at various levels of cardiovascular risk classified according to the SCORE risk chart.**

	VHR	**MR**	**LR**	***p-value (non-adjusted)***	***p value (adjusted)****
Number of participants	229	32	81		
TNFα plasma levels (pg/ml)	4.711 ± 2.453	3.056 ± 0.767 *(n=31)*	2.658 ± 0.729 *(n=80)*	p<0.0001	0.003
TACE* mRNA levels/GAPDH *(2^(-DcT))	0.05366 ± 0.03127 (n=225)	0.03818 ± 0.02501	0.03739 ± 0.01881 (n=81)	p<0.0001	0.031
TACE plasma levels (pg/ml)	739.74 ± 2738.67	566.52 ± 1405.83	1013.87 ± 4369.61	0.087	0.924
	VHR	**Non-VHR**		***p-value (non-adjusted)***	***p value (adjusted)****
Number of participants	229	115			
TNFα plasma levels (pg/ml)	4.711 ± 2.453	2.770 ± 0.752 *(n=113)*		p<0.0001	<0.0001
TACE* mRNA levels/GAPDH *(2^(-DcT))	0.05366 ± 0.03127 *(n=225)*	0.03755 ± 0.*02051 (n=114)*		p<0.0001	0.006
TACE plasma levels (pg/ml)	739.74 ± 2738.67	905.90 ± 3750.23		0.044	0.642

ANCOVA analysis was used to compare the cohorts. Two p values were calculated to assess the impact of the covariates on the analysis. (LR: low risk; MR: moderate risk; non-VHR: non-very high risk; SCORE: Systematic COronary Risk Evaluation risk score; TACE: tumour necrosis factor alpha converting enzyme, TNFα: tumour necrosis factor alpha; VHR: very high risk

**adjusted for age and gender for TNFα plasma levels, TACE gene expression and TACE plasma levels. For statistical analysis TNFα plasma levels, TACE gene expression and TACE plasma levels were transformed using a log transformation as values did not follow the normal distribution*

3.3.2 TNFα plasma levels were higher in VHR participants

To determine whether TNFα levels were different in individuals at various levels of cardiovascular risk, TNFα soluble levels were measured in the plasma. Results showed that TNFα plasma levels were significantly higher in VHR compared to non-VHR participants (4.711 ± 2.453 pg/ml *vs.* 2.770 ± 0.752 pg/ml; p<0.0001) (Figure 1a) corroborating previous work (181). Furthermore, TNFα plasma levels increased

significantly with increasing cardiovascular risk with the highest levels in the VHR category and the lowest levels in the LR category ($p<0.0001$) (Figure 1b and Table 3).

To investigate whether TNFα plasma levels could further stratify the VHR population, various subgroups were compared. TNFα plasma levels were higher in VHR participants who had experienced a previous myocardial infarction (MI) compared to those that had not experienced a previous MI (5.399 ± 2.368 pg/ml *vs.* 4.389 ± 2.433 pg/m; $p<0.01$) (Figure 1c). To explore whether previous MI stratified both ACS-VHR and ELEC-VHR, TNFα plasma levels were compared in ACS-VHR and ELEC-VHR. The present data showed that TNFα plasma levels were also significantly higher in ACS-VHR participants with a previous MI compared to ACS-VHR participants with no previous MI (5.919± 3.028 pg/ml vs. 4.401± 2.609 pg/ml; $p<0.05$) and in ELEC-VHR participants with a previous MI compared to ELEC-VHR participants with no previous MI (5.035± 1.720 pg/ml vs. 4.355 ± 2.109 pg/ml; $p<0.05$).

In terms of revascularisation, TNFα plasma levels were higher in VHR participants who had underwent a previous percutaneous coronary intervention (PCI) compared to VHR participants who had not undergone a previous PCI (5.260 ± 2.337 pg/ml *vs.* 4.454 ± 2.471 pg/ml; $p<0.01$) (Figure 1d). This difference was also observed in ACS-VHR participants with a previous PCI compared to ACS-VHR participants with no previous PCI (5.707 ± 3.106 pg/ml vs. 4.500 ± 2.643 pg/ml; $p<0.05$) and in ELEC-VHR participants with a previous PCI compared to ELEC-VHR participants with no previous PCI (4.982 ± 1.675 pg/ml vs. 4.374 ± 2.159 pg/ml; $p<0.05$). Interestingly, however, TNFα plasma levels were not higher in participants with a previous coronary artery

bypass surgery (CABG). In addition, TNFα plasma levels were not significantly different between ACS-VHR and ELEC-VHR individuals.

VHR participants with a previous MI and a previous PCI were both more likely to be on statin (90.4%; $p<0.0001$ and 91.8% $p<0.0001$ respectively), anti-platelet (90.4%; $p<0.0001$ and 91.8%; $p<0.0001$ respectively) and anti-hypertensive therapy (90.4%; $p<0.0001$ and 93.2%; $p<0.0001$ respectively) and had both lower levels of total cholesterol (3.76 ± 0.96 vs. 4.38 ± 1.41; $p<0.01$ and 3.73 ± 1.00 vs. 4.41 ± 1.40; $p<0.001$ respectively) compared to those with no previous MI or a previous PCI.

In terms of first cardiovascular event and recurrent MACE, results showed that TNFα plasma levels were higher in ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first event (5.594 ± 3.878 vs. 4.114 ± 1.635; $p<0.01$) (Figure 1e).

Regarding VHR participants with different comorbidities, TNFα plasma levels were higher in VHR participants with diabetes (19.2%) compared to non-diabetics (5.328 ± 1.958 vs. 4.564 ± 2.539; $p<0.01$). TNFα plasma levels were also higher in VHR participants with hypertension (55.9%) compared to VHR participants with no hypertension (5.161 ± 2.851 vs. 4.141 ± 1.676; $p<0.05$). Furthermore, TNFα plasma levels were higher in VHR participants with a diagnosis of heart failure (15.7%) (5.320 ± 2.191 vs. 4.600 ± 2.489; $p<0.05$) compared to those with no diagnosis of heart failure. In addition, TNFα plasma levels were higher in VHR individuals with arthritis compared to those with no arthritis (5.790 ± 3.578 vs. 4.517 ± 2.146; $p<0.01$) and in VHR participants with glomerular filtration rate (GFR) of less than 60 ml/min compared to those with a GFR higher than 60 ml/min (5.731 ± 2.62 vs. 4.50 ± 2.36;

p<0.05). Interestingly, participants with a GFR of less than 60 ml/min were more likely to be ACS-VHR (p<0.05).

Moreover, TNFα plasma levels were higher in VHR participants on statin (4.972 ± 2.625 vs. 4.079 ± 1.795; p<0.01), anti-platelet (5.085 ± 2.685 vs. 3.975 ± 1.660; p<0.01) and anti-hypertensive therapy (5.007 ± 2.630 vs. 3.856 ± 1.520; p<0.01).

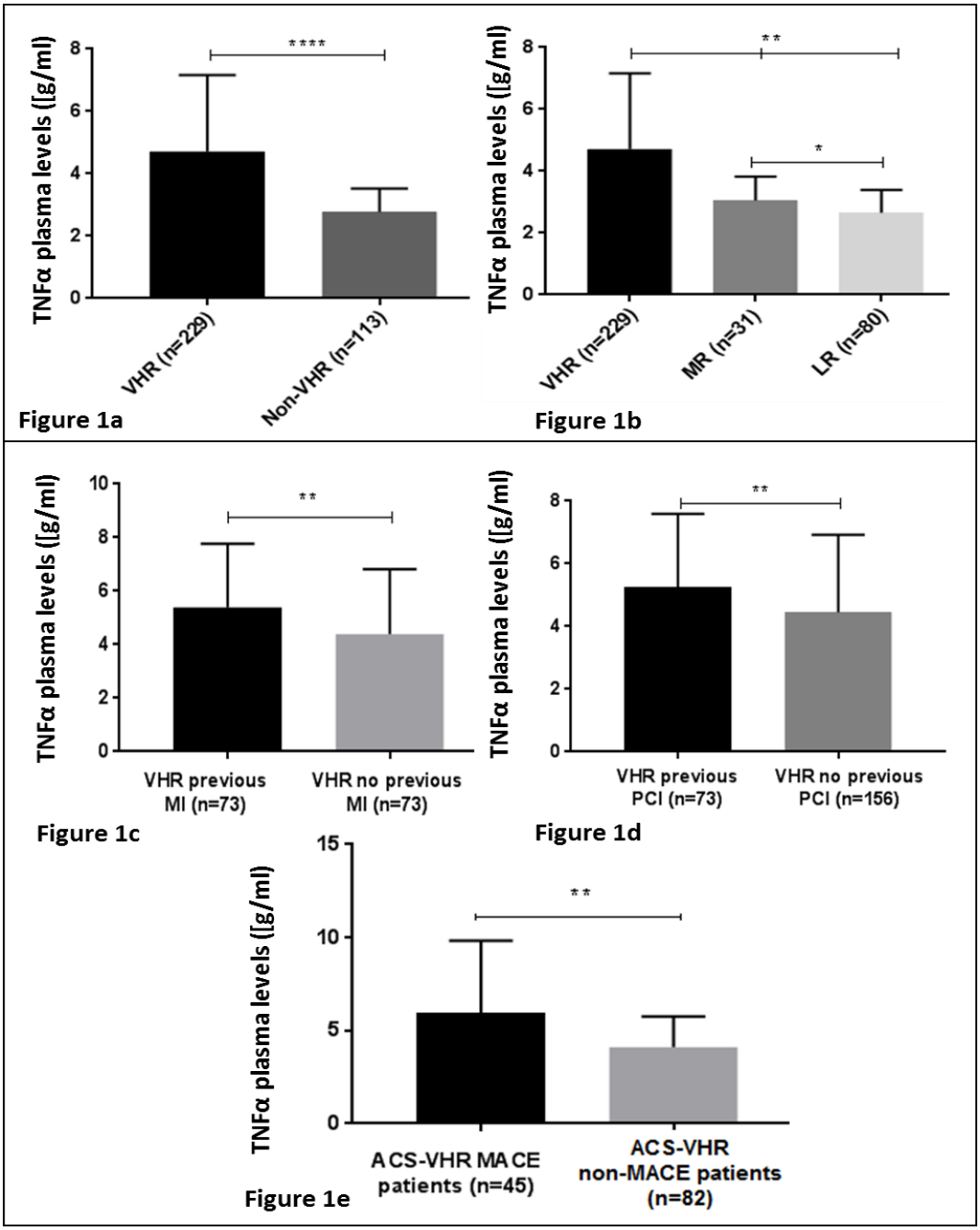


Figure 1: ***TNFα plasma protein levels are higher in participants at very high cardiovascular risk.*** *TNFα plasma protein levels were measured by ELISA using the MSD® MULTI-SPOT Assay System. Figure 1a: TNFα plasma levels are higher in VHR participants compared to non-VHR participants. Figure 1b: TNFα plasma levels are higher with increasing cardiovascular risk. Figure 1c: TNFα plasma levels are higher in VHR participants with previous MI compared to VHR participants with no previous MI. Figure 1d: TNFα plasma levels are higher in VHR participants with previous PCI compared to VHR participants with no previous PCI. Figure 1e: TNFα plasma levels in ACS-VHR participants admitted for MACE. (ACS: acute coronary syndrome; LR: low risk; MACE: Major adverse cardiovascular events; MI: myocardial infarction; MR: moderate risk; non-VHR: non-very high risk participants; PCI: Percutaneous coronary intervention; TNFα: tumour necrosis factor alpha; VHR: non-very high risk;*

*VHR: very high risk). Statistical analysis was performed by ANCOVA. **p<0.01; ****p<0.0001*

3.3.3 *TACE* mRNA levels were higher in VHR participants

To explore whether *TACE* gene expression was associated with cardiovascular risk, *TACE* mRNA levels were measured in peripheral blood mononuclear cells (PBMCs). Results demonstrated that *TACE* mRNA levels were higher in VHR participants compared to non-VHR participants (0.05366 ± 0.03127 vs. 0.03755 ± 0.02051; p<0.01) (Figure 2a). In addition, *TACE* mRNA levels increased significantly with increasing cardiovascular risk where the expression was highest in the VHR category and the lowest in the LR category (p<0.05) (Figure 2b and Table 3).

To investigate whether *TACE* gene expression could further stratify the VHR population, various subgroups were compared. Results indicate that *TACE* mRNA levels were lower in VHR participants who had underwent previous PCI compared to VHR participants who had not undergone a previous PCI (0.04624 ± 0.02494 vs. 0.05708 ± 0.03331 with p<0.05) (Figure 2c). In relation to whether previous PCI differentiated between both ACS-VHR and ELEC-VHR participants, results showed that *TACE* mRNA levels tended to be lower in ACS-VHR individuals with a previous PCI compared to ACS-VHR individuals with no previous PCI (0.0476 ± 0.02558 vs. 0.05615 ± 0.03119; p=0.117) and in ELEC-VHR with a previous PCI compared to ELEC-VHR participants with no previous PCI (0.04535 ± 0.02478 vs. 0.05868 ± 0.03690; p=0.054). However, *TACE* mRNA levels were not significantly different between ACS-VHR and ELEC-VHR individuals.

VHR participants with a previous PCI were more likely to be on statin (91.8% p<0.0001), anti-platelet (91.8%; p<0.0001) and anti-hypertensive therapy (93.2%; p<0.0001) and had lower levels of total cholesterol (3.73 ± 1.00 vs. 4.41 ± 1.40; p<0.001).

3.3.4 Cell membrane bound TACE protein levels were higher in VHR participants

After measuring *TACE* gene expression in the PBMCs, one could speculate whether cell membrane bound TACE protein levels were also associated with cardiovascular risk. Therefore, cell membrane bound TACE protein levels were measured in the PBMCs in a random sample of participants. Data showed that cell membrane bound TACE protein levels were higher in VHR (n=80) *vs.* non-VHR (n=36) participants (383.9 ± 259.6 pg/ml vs. 221.9 ± 78.8 pg/ml with p<0.0001) (Figure2d). Additionally, cell membrane bound TACE protein levels appeared to be higher in MR vs LR participants (275.5 ± 86.36 pg/ml vs 209.71 ±73.25 pg.ml with p=0.057). Cell membrane bound TACE levels were not statistically different in ACS-VHR compared to ELEC-VHR participants. However, cell bound TACE levels were higher in ACS-VHR admitted for a first cardiovascular event compared to non-VHR participants (415.89 ± 294.60 vs 209.71 ± 73.25, p<0.01). Moreover, cell membrane bound TACE protein levels were not statistically different between VHR participants with a previous MI compared to those with no previous MI or in VHR participants with a previous PCI compared to VHR participants with no previous PCI.

3.3.5 TACE plasma levels were not significantly different between VHR and non-VHR participants

To test if TACE protein is detectable in the plasma and whether levels are associated with cardiovascular risk, TACE plasma levels were measured. Results demonstrated that TACE plasma levels were only significantly different between VHR and non-VHR participants before adjustment ($p<0.05$) but the statistical significance was lost after adjustment for age and gender ($p= 0.642$) (Figure 2e and Table 3) where age was a strong confounder. Furthermore, TACE plasma levels were not statistically different in ACS-VHR compared to ELEC-VHR participants or in VHR participants with a previous MI compared to those with no previous MI or in VHR participants with a previous PCI compared to those with no previous PCI.

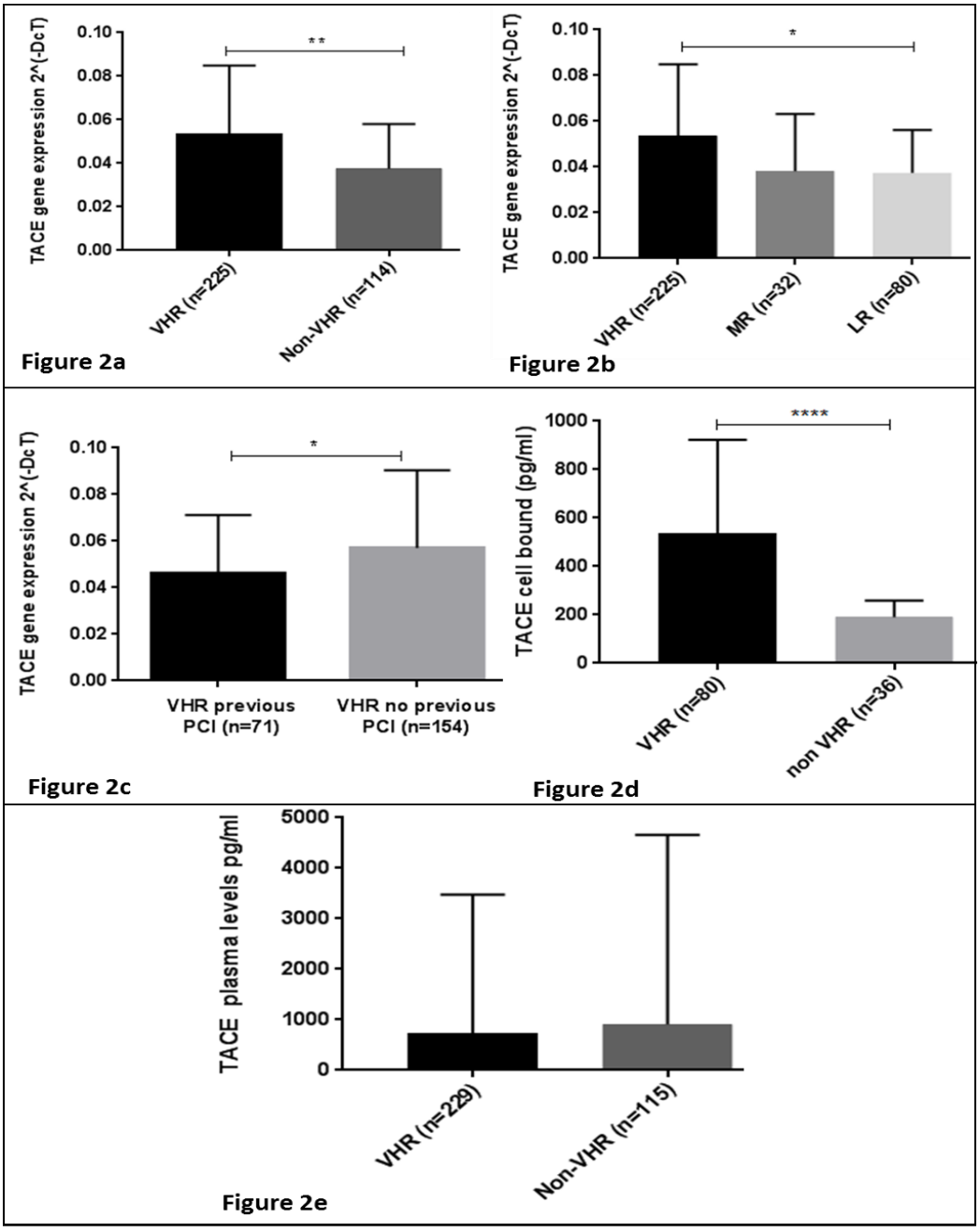


Figure 2: **TACE** ***gene expression, protein expression and plasma levels in participants at various levels of cardiovascular risk.*** *TACE gene expression was measured in the peripheral blood mononuclear cells (PBMCs) by quantitative real-time PCR and normalised to GAPDH. Cell membrane bound TACE protein levels were measured by ELISA following a total protein extraction from the PBMCs after normalisation to 5000ug/ml of total protein. TACE plasma protein levels were measured by ELISA. Figure 2a:* TACE *gene expression is higher in VHR compared to non-VHR participants. Figure 2b:* TACE *gene expression is higher with increasing cardiovascular risk. Figure 2c:* TACE *gene expression is higher in VHR participants with previous PCI compared to VHR participants without a previous PCI. Figure 2d: Cell membrane bound TACE protein levels are higher in VHR compared to non-VHR participants. Figure 2e: TACE plasma protein levels in VHR compared to non-VHR*

*participants. (LR: low risk; MR: moderate risk; non-VHR: non-very high risk participants; PCI: Percutaneous coronary intervention; TACE: tumour necrosis factor alpha converting enzyme; VHR: very high risk). Statistical analysis was performed by ANCOVA. *p<0.05, **p<0.01, ****p<0.0001*

3.3.6 CRP plasma levels did not further differentiate between VHR participant sub-groups

To investigate whether TNFα and TACE have a superior value to CRP measurement in VHR individuals, CRP levels were compared across various VHR subgroups. Results showed that CRP plasma levels were not significantly different between ACS-VHR compared to ELEC-VHR participants (p=0.942). Furthermore, CRP plasma levels were not different between VHR participants with a previous MI compared to those with no previous MI (p=0.942) or in participants with a previous PCI compared to those with no previous PCI (p=0.887). Additionally, CRP plasma levels were not significantly different between VHR diabetic participants compared to VHR non-diabetic participants (p=0.286) or between VHR participants with heart failure compared to VHR participants with no heart failure (p=0.815). Moreover, CRP plasma levels were not significantly different between VHR participants with a GFR less than 60 ml/min compared to those with a GFR higher than 60 ml/min (p=0.475). CRP plasma levels were also not significantly different between VHR participants admitted for a recurrent MACE compared to those admitted for their first event (p=0.294).

3.3.7 *TACE* gene expression correlated positively with TNFα plasma levels and both proteins can predict cardiovascular risk

To explore whether TNFα and TACE levels correlated with different variables explored in the study, a correlation analysis was performed (section 3.2.7). *TACE*

mRNA levels correlated positively with TNFα plasma levels, age and body mass index (BMI) ($p<0.01$, $p<0.001$ and $p<0.05$ respectively) and TACE plasma levels correlated negatively with age ($p<0.05$). TNFα plasma levels correlated positively with C reactive protein (CRP) plasma levels, age and BMI ($p<0.01$, $p<0.0001$ and $p<0.05$ respectively) and negatively with cholesterol levels ($p<0.0001$). Refer to Table 5 for full correlation matrix and p values.

To investigate whether a panel combining TNFα and TACE mRNA levels could significantly predict the presence of a cardiovascular risk, a logistic regression analysis was performed (section 3.2.7). Results indicated that *TACE* gene expression and TNFα plasma levels could collectively predict the cohort membership (VHR or non-VHR) of 79.6% of the participants ($p<0.0001$).

<u>Table 4:</u> Correlation matrix table between TACE, TNFα, CRP, age, BMI, systolic BP and total cholesterol levels

		TACE plasma levels (pg/ml)	*TACE* mRNA levels/ GAPDH *(2^(-DcT))*	TNFα plasma levels (pg/ml)	CRP levels (mg/L)	Age (years)	BMI (Kg/m 2)	Systolic BP (mmHg)	Total cholesterol (mmol/L)
TACE plasma levels (pg/ml)	Pearson Correlation	1	-.052	-.101	-.003	-.130*	-.070	-.046	.051
	Sig. (2-tailed)		.348	.064	.961	.017	.219	.437	.373
	N	336	331	334	286	336	312	288	311
TACE* mRNA levels/GAPDH *(2^(-DcT))	Pearson Correlation	-.052	1	.162**	.105	.208**	.112	.100	-.085
	Sig. (2-tailed)	.348		.003	.077	.000	.050	.092	.138
	N	331	331	329	283	331	308	283	306
TNFα plasma levels (pg/ml)	Pearson Correlation	-.101	.162**	1	.186**	.470**	.126*	.109	-.272**
	Sig. (2-tailed)	.064	.003		.002	.000	.026	.067	.000
	N	334	329	334	284	334	310	286	309
CRP levels (mg/L)	Pearson Correlation	-.003	.105	.186**	1	.100	.115	-.114	-.116
	Sig. (2-tailed)	.961	.077	.002		.091	.062	.076	.059
	N	286	283	284	286	286	264	243	264
Age (years)	Pearson Correlation	-.130*	.208**	.470**	.100	1	.080	.205**	-.307**
	Sig. (2-tailed)	.017	.000	.000	.091		.159	.000	.000
	N	336	331	334	286	336	312	288	311
BMI (Kg/m2)	Pearson Correlation	-.070	.112	.126*	.115	.080	1	.099	-.016
	Sig. (2-tailed)	.219	.050	.026	.062	.159		.103	.783
	N	312	308	310	264	312	312	271	289
Systolic BP (mmHg)	Pearson Correlation	-.046	.100	.109	-.114	.205**	.099	1	.074
	Sig. (2-tailed)	.437	.092	.067	.076	.000	.103		.224
	N	288	283	286	243	288	271	288	268
Total cholesterol (mmol/L)	Pearson Correlation	.051	-.085	-.272**	-.116	-.307**	-.016	.074	1
	Sig. (2-tailed)	.373	.138	.000	.059	.000	.783	.224	
	N	311	306	309	264	311	289	268	311

Highlighted cells refer to a significant result. (BMI: body mass index; BP: blood pressure; CRP: C-reactive protein; TACE: tumour necrosis factor alpha converting enzyme, TNFα: tumour necrosis factor alpha). Pearson's correlation was used to evaluate the correlation between variables.

3.3.8 TACE and TNFα plasma levels did not correlate with angiographic outcome

To explore if TNFα and TACE levels could determine the plaque burden in VHR participants, a multinomial logistic regression analysis was carried out (section 3.2.7). This showed that the measured biomarkers were not able to effectively predict the angiographic outcome and the severity of the plaque burden. In addition, an ANOVA analysis did not show any difference between the biomarker levels and the different categories of angiographic outcome.

<u>Table 5:</u> TNFα plasma and TACE plasma and gene expression levels in very high risk participants.

	TACE plasma levels (pg/ml)	***p value***	***TACE* mRNA levels /GAPDH *(2^(-DcT))***	***p value***	**TNFα plasma levels (pg/ml)**	***p value***
Demographic and History *(n; %)*						
Gender						
Male *(180; 78.6)*	811.067 ± 3019.50	0.9640	0.5453 ± 0.319	0.4470	4.655 ± 2.489	0.3370
Female *(49; 21.4)*	477.741 ± 1239.84		0.5040 ± 0.028		4.9169 ± 2.33	
Diabetes mellitus						
Present *(44; 19.2)*	815.13 ± 3431.31	0.194	0.05685 ± 0.02261	0.102	5.328 ± 1.958	0.007
Absent *(185; 80.8)*	721.81 ± 2557.47		0.05290 ± 0.03299		4.564 ± 2.539	
Heart failure						
Present *(36; 15.7)*	268.44 ± 634.12	0.349	0.05500 ± 0.03040	0.668	5.320 ± 2.191	0.042
Absent *(193; 84.3)*	827.65 ± 2963.77		0.05340 ± 0.01315		4.600 ± 2.489	
Renal function						
GFR < 60 *(45; 20)*	1053.18 ± 4282.93	0.347	0.0550 ± 0.0303	0.778	5.731 ± 2.62	0.02
GFR > 60 *(180; 80)*	675.50 ± 2237.94		0.0534 ± 0.0317		4.50 ± 2.36	
Hypertension						
Present *(128; 58.9)*	783.59 ± 3247.21	0.963	0.05455 ± 0.03378	0.918	5.161 ± 2.851	0.021
Absent *(101; 44.1)*	684.17 ± 1924.52		0.05251 ± 0.02789		4.141 ± 1.676	
Dyslipidaemia						
Present *(134; 58.5)*	812.73 ± 3262.10	0.775	0.05426 ± 0.03133	0.723	4.613 ± 1.752	0.714
Absent *(95; 41.5)*	636.80 ± 1765.69		0.05281 ± 0.03133		4.850 ± 3.200	
Arthritis						
Present *(35; 15.3)*	612.42 ± 1512.25	0.601	0.05374 ± 0.03152	0.975	5.790 ± 3.578	0.003
Absent *(194; 84.7)*	762.71 ± 2907.60		0.05364 ± 0.0313		4.517 ± 2.146	
Depression						
Present *(43; 18.8)*	484.06 ± 1379.08	0.906	0.04754 ± 0.02404	0.396	4.784 ± 1.835	0.067
Absent *(186; 81.2)*	798.85 ± 2965.32		0.05510 ± 0.03264		4.694 ± 2.579	
Present or previous cancer						
Present *(27; 11.8)*	1450.02 ± 5478.88	0.428	0.06442 ± 0.04069	0.108	5.243 ± 2.178	0.507

Absent *(202; 88.2)*	644.80 ± 2132.61		0.05219 ± 0.02958		4.640 ± 2.484	
Smoking status						
None *(66; 30.3)*	820.51 ± 2134.71	0.21	0.0512 ± 0.026	0.253	4.584 ± 1.736	0.58
Ex-smoker *(110; 50.4)*	843.83 ± 3510.60		0.0596 ± 0.0364		5.020 ± 2.875	
Current *(42; 19.3)*	311.69 ± 897.17		0.0464 ± 0.0229		4.342 ± 2.390	
Pharmacotherapy						
Statin therapy						
Present *(165; 72.7)*	753.25 ± 3020.58	0.941	0.05284 ± 0.03016	0.828	4.972 ± 2.625	0.002
Absent *(62; 27.3)*	641.31 ± 1778.00		0.05603 ± 0.03437		4.079 ± 1.795	
Antiplatelet therapy						
Present *(154; 67.8)*	755.30 ± 3102.58	0.559	0.05266 ± 0.03013	0.654	5.085 ± 2.685	0.002
Absent *(73; 32.1)*	653.87 ± 1733.81		0.05592 ± 0.03380		3.975 ± 1.660	
Antihypertensive therapy						
Present *(172; 76.8)*	712.93 ± 2943.38	0.984	0.05379 ± 0.03125	0.881	5.007 ± 2.630	0.005
Absent *(55; 24.2)*	753.18 ± 1963.55		0.05354 ± 0.03190		3.856 ± 1.520	
Antianginal therapy						
Present *(88; 38.8)*	740.57 ± 3196.08	0.865	0.05083 ± 0.03113	0.284	5.104 ± 2.510	0.033
Absent *(139; 61.2)*	711.36 ± 2409.20		0.05551 ± 0.03145		4.490 ± 2.399	
Diuretic therapy						
Present *(56; 24.7)*	848.74 ± 3066.91	0.69	0.06210 ± 0.03555	0.034	5.547 ± 3.260	0.023
Absent *(171; 75.3)*	681.40 ± 2624.79		0.05099 ± 0.02944		4.460 ± 2.070	
Drug naïve						
Present *(54; 23.7)*	816.63 ± 1980.84	0.339	0.05572 ± 0.03350	0.663	3.926 ± 1.575	0.033
Absent *(174; 76.3)*	690.22 ±2926.30		0.05317 ± 0.03062		4.960 ± 2.629	
Clinical Variables						
ACS-VHR *(127; 55.5)*	789.71 ± 2879.24	0.464	0.05425 ± 0.03015	0.64	4.766 ± 2.785	0.829
ELEC-VHR *(102; 44.5)*	677.52 ± 2565.61		0.05289 ± 0.03277		4.642 ± 1.975	
Previous MI						
Present *(73; 31.9)*	1027.00 ± 4219.04	0.647	0.05166 ± 0.02748	0.588	5.399 ± 2.368	0.01
Absent *(156; 68.1)*	605.31 ± 1645.34		0.05456 ± 0.03288		4.389 ± 2.433	

Previous MI ACS participants						
Present (30; 23.6)	1255.74 ± 5021.08	0.917	0.05384 ± 0.02278	0.775	5.919± 3.028	0.020
Absent (97; 76.4)	645.58 ± 1618.65		0.05437 ± 0.03213		4.401± 2.609	
Previous MI ELEC participants						
Present (43; 42.1)	867.43 ± 3431.18	0.466	0.05011 ± 0.05035	0.367	5.035± 1.720	0.023
Absent (59; 57.8)	539.11 ± 1700.24		0.05486 ± 0.03438		4.355 ± 2.109	
Previous PCI						
Present *(73; 31.9)*	857.50 ± 3485.28	0.741	0.04624 ± 0.02494	0.011	5.260 ± 2.337	0.009
Absent *(56; 68.1)*	684.65 ± 2319.61		0.05708 ± 0.03331		4.454 ± 2.471	
Previous PCI ACS participants						
Present (28; 22.0)	1369.63 ± 5354.52	0.527	0.0476 ± 0.02558	0.117	5.707 ± 3.106	0.028
Absent (99; 78.0)	625.70 ± 1623.64		0.05615 ± 0.03119		4.500 ± 2.643	
Previous PCI ELEC participants						
Present (45; 44.1)	538.81 ± 1418.77	0.913	0.04535 ± 0.02478	0.054	4.982 ± 1.675	0.028
Absent (57; 55.9)	787.03 ± 3203.51		0.05868 ± 0.03690		4.374 ± 2.159	
Previous CABG						
Present *(22; 9.6)*	743.22 ± 2100.94	0.999	0.05980 ± 0.03212	0.188	6.005 ± 4.441	0.102
Absent *(207; 90.4)*	739.37 ± 2802.03		0.05299 ± 0.03118		4.573 ± 2.110	
Previous MI, PCI or CABG						
Present *(100; 43.7)*	1041.73 ± 3806.48	0.356	0.05128 ± 0.02863	0.345	5.380 ± 2.905	0.001
Absent *(129; 56.3)*	505.65 ± 1423.70		0.05546 ± 0.03312		4.192 ± 2.905	
Diagnosis upon admission						
Stable Angina (85; 37.1)	681.56 ± 2753.69	0.106	0.0557 ± 0.0341	0.744	4.656 ± 2.080	0.602
Unstable Angina (21; 9.2)	1778.32 ± 6161.32		0.0559 ± 0.0321		4.476 ± 3.314	

NSTEMI < 1 week (77; 33.6)	472.89 ± 1040.14		0.0559 ± 0.0306		4.835 ± 2.949	
STEMI < 1 week (7; 3.0)	96.30 ± 177.65		0.0521 ± 0.444		4.867 ± 1.888	
Other (39; 17.03)						
ACS-VHR participants admitted for MACE						
Present *(45; 35.4)*	1045.36 ± 4259.01	0.28	0.0550 ± 0.0300	0.999	5.594 ± 3.878	0.007
Absent *(82; 64.6)*	649.42 ± 1727.99		0.0538 ± 0.0304		4.114 ± 1.635	
Participants with no previous MI, PCI or CABG						
ACS-VHR *(84, 65.1)*	637.52 ± 1708.80	0.56	0.00538 ± 0.03951	0.877	4.179 ± 1.686	0.542
ELEC-VHR *(45, 34.9)*	259.48 ± 541.41		0.05865 ± 0.03778		4.217 ± 2.240	
Prognostic variable						
Follow-up MACE						
Within 6 months *(5; 2.2)*	99.68 ± 161.66	0.801	0.0495 ± 0.0258	0.798	6.118 ± 0.716	0.292
After 6 months *(11; 4.8)*	2096.00 ± 6725.63		0.4814 ± 0.0308		5.022 ± 2.246	
Absent *(213; 93.0)*	684.72 ± 2413.31		0.5404 ± 0.3150		4.662 ± 2.483	

Highlighted cells refer to p values <0.05 (ACS: acute coronary syndrome participants; CABG: Coronary artery bypass surgery; ELEC: elective participants; GFR: Glomerular filtration rate; MACE: major adverse cardiovascular events MI: Myocardial Infarction; NSTEMI: non-ST elevation myocardial infarction; PCI: percutaneous coronary intervention; STEMI: ST elevation myocardial infarction; TACE: tumour necrosis factor alpha converting enzyme; TNFα: tumour necrosis factor alpha; VHR: very high risk).

3.4 Discussion

The results of this study demonstrated that TNFα and TACE could be potential markers for CVD risk. In addition, TNFα and TACE were able to further stratify individuals within the VHR group who were at higher risk of MACE with a higher sensitivity compared to CRP.

3.4.1 TNFα plasma and *TACE* mRNA levels identified and further stratified VHR participant sub-groups

This study demonstrated that TNFα plasma levels were higher in VHR participants compared to non-VHR individuals corroborating previous results (153,154,181). Additionally, TNFα plasma levels were sufficiently sensitive in identifying cardiovascular risk in parallel to the SCORE risk which is a novel finding. Interestingly, in this study, TNFα plasma levels were higher in VHR participants with a previous MI compared to VHR participants with no previous MI and in VHR participants with previous PCI compared to VHR participants with no previous PCI. This was observed in both ACS-VHR and ELEC-VHR individuals. Participants with a previous MI and PCI were more likely to be on statin, antiplatelet and antihypertensive therapy. This indicates that, in these participants, medical management and revascularisation strategies fail to lower TNFα plasma levels post-MI and post-PCI. Persistent high TNFα plasma levels post-MI were attributed to endothelial dysfunction in diabetic patients with a previous MI (182) and it was shown that statins can protect the endothelial cells from TNFα induced inflammation (183). However, many studies have discussed the controversial effects of inhibiting TNFα in CVD and suggested that minimal levels of TNFα are cardioprotective (184). The predictive value of TNFα plasma levels in

relation to the occurrence of cardiovascular events has been previously reported (185). In agreement with these results, the current study shows that TNFα plasma levels were higher in ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first cardiovascular event. Persistently elevated TNFα plasma levels post-MI have been linked to an increased risk of MACE (181). These results suggest that although TNFα might not be an ideal target in CVD, it could be used as a potential biomarker for recurrent MACE. Interestingly, TNFα plasma levels were not significantly higher in VHR participants with a previous CABG compared to those with no previous CABG. This demonstrates the previously reported superior value of CABG as a revascularisation strategy as opposed to PCI (186).

In view of the previous results, levels of TACE, the enzyme responsible for TNFα cleavage, were measured. This was to explore whether TACE levels could add value to the CVD risk assessment. Results showed that, similarly to TNFα plasma levels, *TACE* gene expression was higher in VHR compared to non-VHR participants. Interestingly, in contrast to what was observed with TNFα plasma levels, *TACE* gene expression was lower in VHR participants with a previous PCI compared to VHR participants with no previous PCI. This trend was also observed in ACS-VHR and ELEC-VHR individuals. Since participants with a previous PCI were on statin, antiplatelet and antihypertensive therapy, this highlights the possible effect of medical management and revascularisation strategies on lowering *TACE* gene expression. Nevertheless, it appears that after a PCI and the initiation of a post-PCI medical therapy, despite a decrease in *TACE* gene expression, TNFα plasma levels remain increased. This increase in TNFα plasma levels could be due to different activators

that, when present, could enhance TNFα shedding (187), and moreover, there is evidence that TNFα could be cleaved by other sheddase such as ADAM10 (188) matrix metalloproteinase 7 (189) or matrix metalloproteinase 13 (190). Additionally, circulating TNFα might not be biologically active as it might be captured by circulating tumour necrosis factor alpha 1 (TNFR1) and 2 (TNFR2) soluble receptors (191). It is noteworthy to mention that membrane cholesterol depletion has been shown to increase TACE shedding activity and could explain the observed higher TNFα plasma levels following statin treatment post MI and post PCI (192,193). On the other hand, TACE is known to regulate angiogenesis in cardiomyocytes following MI and when upregulated within the normal range, is suggested to provide protective effects post-MI (194). In the present study, *TACE* downregulation in participants with a previous PCI could be associated with adverse outcomes. However, the mechanisms behind this effect need further understanding.

However, unlike TNFα levels and *TACE* gene expression, CRP plasma levels were not able to identify VHR participants with a previous MI or a previous PCI. This strengthens the value of TNFα and TACE in potentially characterising different subgroups of VHR individuals with CVD. In addition, CRP plasma levels were not able to identify VHR participants with diabetes, heart failure or renal failure. This provides additional evidence of the superior role of TNFα and TACE in further stratifying VHR individuals. The value of combining TNFα and TACE measurement in a possible biomarker screening panel for primary prevention of a CVD event was reflected in a logistic regression model. This panel was able to successfully assign participants into either VHR or non-VHR group in 79.6% of the cases ($r=0.449$ and $p<0.0001$). However, the practicality of measuring gene expression levels in a biomarker panel requires

optimisation and the measurement of protein levels, when possible, might be more practical (137).

3.4.2 Exploration of TACE plasma and cell membrane bound levels in individuals at various levels of cardiovascular risk

In biomarker development, it is essential for the marker to be measured quickly and effectively so the results can orient a patients' clinical management upon admission. As measuring *TACE* mRNA levels requires different steps before obtaining a result, this could delay admission and initiation of an appropriate therapy. Therefore, two alternative strategies to TACE measurement were explored. The aim of the first strategy was to investigate whether TACE plasma levels were detectable in participants with CVD and whether plasma levels showed a similar pattern to TNFα plasma and *TACE* mRNA levels. To date, this is the first study to measure active TACE plasma levels in a cohort with CVD. Interestingly, TACE plasma levels showed a completely opposite trend compared to TNFα and *TACE* gene expression levels. TACE plasma levels appeared to be lower in VHR compared to non-VHR participants but failed to reach statistical significance when controlled for age and gender. However, it is noteworthy that TACE plasma levels were hardly detectable in some participants and were extremely high in other participants which highlights a need for further assay development.

These results demonstrated that TACE protein can be detected in the plasma which has also been proven in other studies where plasma TACE levels were detected in several inflammatory diseases (176–178) and second, that there is a mechanism behind TACE release in the plasma that needs further investigation. TACE is known

to be a membrane bound protein whose presence on the cell surface is essential in order to cleave the substrates effectively (151,152). Some studies however, have reported the presence of an active form of TACE protein in the plasma (176,177) where its activity has been measured using a Fluorescence Resonance Energy Transfer assay. Nevertheless, whether TACE can effectively cleave distant substrates *in vivo* is unknown. The major known mechanism of TACE elimination from the cell surface is its internalisation (173,174). Although, there is evidence of TACE being detected at the surface of microparticles released from platelets and endothelial cells in the plasma (175,176). Therefore, it appears that there is a mechanism responsible for TACE shedding from the cell surface that could be similar to TACE closest family member ADAM10 which has also been successfully detected in the plasma or serum by ELISA in a previous study (195).

As TACE plasma levels followed a trend where levels were lower in VHR compared to non-VHR participants, it is possible that in VHR participants, TACE might be retained on the cell surface and thus, would exhibit reduced release into the bloodstream. Once retained on the cell surface, TACE would shed a higher number of inflammatory proteins which could aggravate local and systemic inflammation increasing MACE risk in VHR individuals.

To test this hypothesis, a second strategy for TACE measurement was explored. Cell membrane bound TACE protein levels were measured by ELISA after extracting total proteins from a buffy coat preparation and normalising the protein levels before analysis for all participants. The results validated the proposed theory and showed that cell membrane bound TACE levels were higher in VHR compared to non-VHR

participants. Therefore, in VHR participants, following an increase in *TACE* gene expression, TACE protein synthesis is increased. Hence, there is probable a mechanism in place that retains TACE on the cell surface leading to an increase in the shedding of inflammatory transmembrane proteins aggravating local and systemic inflammation in VHR individuals. To study this effect, some of TACE associated proteins could be investigated such as Tissue Inhibitor Of Metalloproteinase 3 (TIMP3) (Refer to Chapter 4) and other proteins that are known to modulate TACE activity such as Four and a half LIM domains protein 2 (FHL2) (196), Synapse-associated protein 97 (SAP97) (197) and Protein tyrosine phosphatase, non-receptor type 3 (PTPH1) (198). Moreover, this data provides preliminary evidence that TACE might be a good target for biological therapeutics. However, new findings demonstrate that TACE pro and anti-atherosclerotic effects are cell specific and a cell targeted approach should rather be considered with regards to TACE inhibition (199–201). This might be possible by developing targeted TACE therapy as it was recently shown in an model of irritable bowel disease (202). Further work is required to fully elucidate the atherogenic effects of TACE. In fact, the authors have shown that a bi-specific inhibitor, that targets cell membrane bound TACE and a pro-inflammatory cytokine, was able to increase its concentration at the cell surface and reduce inflammation in irritable bowel disease. The efficacy of such a promising therapeutical model should be tested in CVD (202).

3.4.3 Strengths and Limitations

Strengths of the study design include the strict plasma sample collection procedure with morning sampling after overnight fasting, the collection of consecutive samples

on recruitment days and the near-complete information on baseline CVD risk factors and follow up events within a year of initial recruitment. Some limitations of the present study should also be acknowledged. The number of participants with follow up MACE events within one year was low, however, participants are still being followed up for MACE events over one year of recruitment. In addition, the definition of MACE in this study was restricted to recurrent ACS, stroke TIA and death. However, several studies include HF and atrial fibrillation (AF) (203,204) in the definition for MACE as well. By including HF and AF in the MACE definition, the total number of patients who develop MACE within a year of admission increases to 13.5% as opposed to 7.0% when HF and AF are not included. Moreover, the number of high risk (HR) participants was only limited to 2 participants and they were therefore included in the non-VHR cohort as the definition of the VHR cohort was restricted to those with evidence of atheroma as per coronary angiogram. In terms of potential biases resulting from the biochemical measurements used in the present study, it is important to consider that TACE plasma levels were below the limit of detection in many participants. We have used a validated TACE ELISA assay (RAB0003 SIGMA) as the other available assays were customised and required a validation by the user (R&D ELISA DuoSet). In addition, *TACE* gene expression could not be measured in 5 participants as RNA was not obtained from those participants and TNFα plasma levels were below the limit of detection in 2 participants and they were therefore excluded from the analysis.

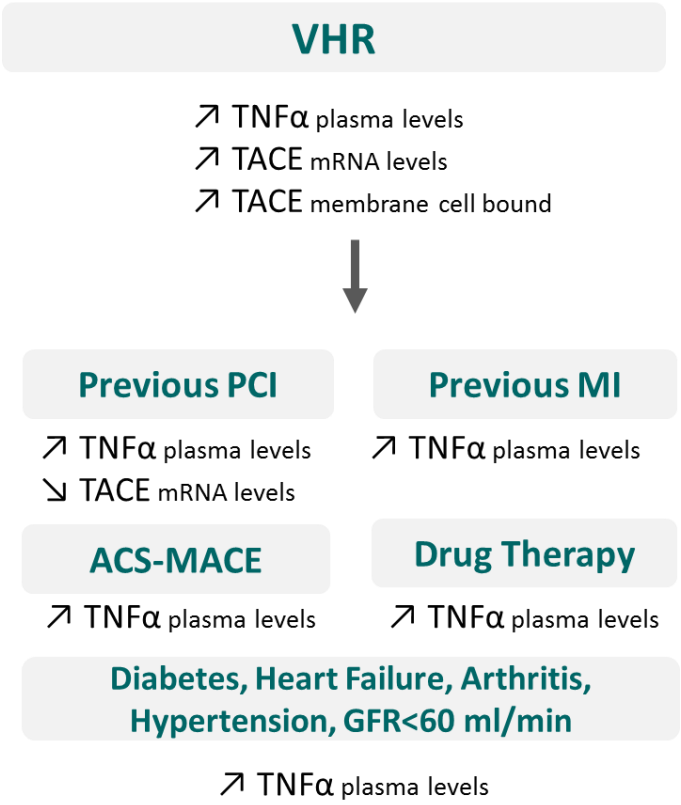


***Figure 3:* Proposed model using TACE and TNFα to identify and further stratify VHR participants.** *(ACS: acute coronary syndrome participants; GFR: Glomerular filtration rate; MACE: major adverse cardiovascular events MI: Myocardial Infarction; PCI: percutaneous coronary intervention; TACE: tumour necrosis factor alpha converting enzyme; TNFα: tumour necrosis factor alpha; VHR: very high risk).*

3.5 Conclusion

In this study, *TACE* gene expression and protein levels (plasma and cell bound form) were measured for the first time in individuals at various levels of cardiovascular risk. Results demonstrate that TACE and TNFα are associated with cardiovascular risk and recurrent events. In addition, after a PCI, TNFα plasma levels remain high whereas TACE mRNA levels are low despite clinical and medical management which underline the need to further investigate the mechanisms behind this process after a PCI. On the other hand, this data provides an insight into the mechanism of action of TACE and highlights the need for future work to further investigate the best way to measure TACE as a marker for CVD risk. This work also provides preliminary evidence of the cell membrane bound form of TACE which may be a valuable target for future therapy development. Additional studies are however required to investigate this and the utility of the TNFα family as biomarkers for predicting MACE risk.

Chapter 4

TNFR1, TNFR2 and TIMP3 identify and further stratify individuals at very high risk of cardiovascular events

Abstract

Background: Inflammation plays a crucial role in cardiovascular disease (CVD). Tumour Necrosis Factor Alpha Converting Enzyme (TACE) is a membrane bound enzyme responsible for cleaving transmembrane Tumour Necrosis Factor Alpha (tmTNFα) and its associated receptors releasing soluble TNFα, Tumour Necrosis Factor Alpha Receptors 1 and 2 (TNFR1 and TNFR2). TNFR1 receptor is a major initiator of inflammation causing endothelial cell dysfunction whereas TNFR2 receptor has more favourable effects by activating angiogenic and survival pathways. Metalloproteinase inhibitor 3 (TIMP3) is the only known endogenous TACE inhibitor. Clinical studies exploring the potential role of such biomarkers in predicting major adverse cardiovascular events (MACE) risk in very high risk (VHR) individuals is lacking. The main purpose of this study was to evaluate whether TNFR1, TNFR2 and TIMP3 levels are associated with cardiovascular risk.

Methods: Participants were recruited from the cardiac catheterisation laboratory or by email advertisement. Group 1 was defined as VHR participants with a 10-year risk SCORE ≥10% risk of fatal CVD. VHR participants were subdivided in Acute Coronary Syndrome (ACS-VHR) and elective percutaneous coronary intervention participants (ELEC-VHR). Group 2 were defined as low, moderate and high risk participants (non-VHR) with a 10-year risk SCORE <10% risk of fatal CVD. TNFR1, TNFR2 and TIMP3 plasma protein levels as well as cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels were measured by enzyme-linked immunosorbent assay (ELISA). *TIMP3* mRNA levels were measured by quantitative real-time PCR.

Results: A total of 344 participants were recruited. The VHR (n=229) group consisted of ACS-VHR (n=127) and ELEC-VHR (n=102) participants. The non-VHR (n=115) group

consisted of low risk (LR) (n=81), moderate risk (MR) (n=32) and high risk (HR) (n=2) participants. TNFR1 plasma protein levels were higher in the VHR compared to the non-VHR group (1291 ± 1158 pg/ml *vs*. 817.4 ± 208.6 pg/ml; p<0.01) and levels were also higher in ACS-VHR *vs*. ELEC-VHR participants (1359 pg/ml ± 775 *vs.* 1103 pg/ml ± 629; p<0.001). TNFR2 plasma protein levels were numerically higher in the VHR compared to the non-VHR group (2839 pg/ml ± 1314 *vs*. 2099 pg/ml ± 620.1; p=0.057) but TNFR2 plasma protein levels were not significantly different in ACS-VHR compared to ELEC-VHR participants (p=0.185). *TIMP3* mRNA levels were lower in the VHR compared to the non-VHR group (0.0001094 ± 0.0001122 *vs.* 0.0001946 ± 0.000305; p<0.05). The ratio of cell membrane bound TNFR2 to TNFR1 protein levels was lower in the VHR compared to the non VHR group (1.127 ± 0.376 *vs.* 1.494 ± 0.604; p<0.05). The ratio of cell membrane bound TIMP3 to TACE protein levels showed that in VHR participants, TACE was not effectively inhibited (3.927 ± 3.064 *vs*. 9.660 ± 5.239; p<0.0001).

Conclusion: TNFR1 and TNFR2 plasma protein levels can identify VHR participants as well as, in the case of TNFR1, discriminate between ACS-VHR and ELEC-VHR participants, which is a novel finding. This is the first report of *TIMP3* downregulation in individuals with CVD. The ratio of cell membrane bound TNFR2 to TNFR1 and TIMP3 to TACE protein levels were also investigated for the first time in CVD individuals and were both lower in the VHR compared to the non-VHR group highlighting a shift towards a pro-inflammatory state in VHR individuals. These proteins should be further investigated as potential biomarkers that may help further stratify VHR individuals at risk of MACE.

4.1 Introduction

Tumour necrosis factor alpha converting enzyme (TACE), also known as ADAM17, is a cell membrane bound protein responsible for the ectodomain shedding of a variety of inflammatory markers, including tumour necrosis factor alpha (TNFα), which play a role in the initiation and progression of cardiovascular disease (CVD) (151,152). TACE is known to shed TNF receptors 1 (TNFR1) and 2 (TNFR2) releasing their soluble forms (151). TNFα superfamily molecules have been linked to CVD and acute coronary syndromes (ACS) in many previous studies (205–207), however, their exploration in individuals with varying levels of cardiovascular risk has not been previously reported.

TNFR1 and TNFR2 are two transmembrane receptors that have opposing effects when it comes to their signalling biology (208). TNFR1 receptor pathways have unfavourable effects, increasing inflammation and causing endothelial cell dysfunction whereas TNFR2 receptor pathways have more favourable effects by activating angiogenic and survival pathways (209,210). Soluble TNFα and transmembrane TNFα (tmTNFα) bind to TNFR1 whereas only tmTNFα is believed to bind to TNFR2 (211). Additionally, TNFR2 has a higher affinity and a longer TNF-binding half-life compared to TNFR1 (212). High circulating levels of TNFR1 have been associated with Parkinson's disease (213) and renal disease (214) whereas high circulating levels of TNFR2 have been linked to rheumatoid arthritis (215) and diabetes (216). Furthermore, high circulating levels of both TNFR1 and TNFR2 soluble receptors have been associated with diabetic complications (206) as well as CVD and comorbidities (207).

Tissue Inhibitor of Metalloproteinase 3 (TIMP3) is the only known endogenous inhibitor of TACE (217) that binds to its catalytic domain in the extracellular matrix (218). TIMP3 was previously reported to prevent inflammation and related metabolic disorders such as insulin resistance and glucose intolerance (219,220) and is downregulated in circulating human monocytes in people at high risk of diabetes (221).

TNFR1, TNFR2 and TIMP3 have been linked separately to CVD, however, clinical studies exploring the potential role of this inflammatory pathway in predicting major adverse cardiovascular events (MACE) in very high risk individuals is lacking. The first aim of this study was to explore any association between TNFR1, TNFR2 and TIMP3 plasma protein levels with CVD risk. The second aim was to investigate whether these biomarker levels are capable of further stratifying individuals at very high risk of MACE.

4.2 Participants and Methods

Refer to **section 2.1** in **Chapter 2** for a detailed description of the participant recruitment process, the risk score used and the methods for blood processing.

4.2.1 Demographic information

Refer to section **3.2.1 in Chapter 3** for a detailed description of the demographic information that was collected in this study.

4.2.3 Measurement of TNFR1, TNFR2 and TIMP3 plasma protein levels

Refer to **section 2.7.1, 2.8.1 and 2.9.1 in Chapter 2.**

4.2.4 Measurement of *TIMP3* gene expression

Refer to **section 2.11, 2.12 and 2.13 in Chapter 2.**

4.2.5 Cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels

Refer to **section 2.4, 2.5, 2.7.2, 2.8.2 and 2.9.2 in Chapter 2.**

4.2.6 Statistical methods

Statistical analysis was carried out as described in **section 3.2.7** in **Chapter 3**

Additionnaly, in this analysis, an inverse function was assigned for TNFR1 and TNFR2 plasma levels variables and a log transformation was applied to the TIMP3 plasma level variables to allow their use in parametric methods of analysis. In the ANCOVA analysis, body mass index (BMI) was controlled for because BMI levels were statistically different between the VHR and the non-VHR group and because BMI levels were associated with the variability in TNFR1 and TNFR2 plasma levels in a linear regression model (180).

4.3. Results

4.3.1 Population Demographics

Refer to section **3.3.1 in Chapter 3** and **Table 1 and 2 in Chapter 3**.

<u>Table 1:</u> TNFR1, TNFR2, TIMP3 and TACE levels in participants at various levels of cardiovascular risk classified according to the SCORE risk chart.

Biomarker type	**VHR**	**MR**	**LR**	***p-value (non-adjusted)***	***p value (adjusted)****
Number of participants	229	32	81		
sTNFR1 plasma levels (pg/ml)	1291 ± 1158	902.4 ± 271	785.1 ± 171.4	p<0.0001	0.036
sTNFR2 plasma levels (pg/ml)	2971.0 ± 2199.0	2349.0 ± 668.30	2015.0 ± 575.0	p<0.0001	0.179
TIMP3 plasma levels (pg/ml)	3397.0 ± 2668.0	3043.0 ± 1615.0	3201.0 ± 1615.0	0.654	0.357
	VHR	**Non-VHR**		***p-value (non-adjusted)***	***p value (adjusted)****
Number of participants	229	115			
sTNFR1 plasma levels (pg/ml)	1291 ± 1158	817.4 ± 208.6		p<0.0001	0.006
sTNFR2 plasma levels (pg/ml)	2971.0 ± 2199.0	2133.0 ± 614.0		p<0.0001	0.057
TIMP3 plasma levels (pg/ml)	3397.0 ± 2668.0	3144.0 ± 2516.0		0.396	0.486
	VHR	**Non-VHR**		***p value***	
TACE cell bound (pg/ml)	383.9 ± 259.6 (n=80)	221.9 ± 78.8 (n=36)		p<0.0001	
TIMP3* mRNA levels/GAPDH *(2^-DcT)	0.0001094 ± 0.0001122 (n=39)	0.0001946 ± 0.000305 (n=20)		0.0317	
TIMP3 cell bound (pg/ml)	1331.13 ± 1101.10 (n=28)	1806.15 ± 1260.91 (n=18)		0.104	
TNFR1 cell bound (pg/ml)	271.71 ± 338.33 (n=23)	126.93 ± 49.08 (n=15)		0.004	
TNFR2 cell bound (pg/ml)	261.82 ± 218.68 (n=23)	185.83 ± 92.32 (n=15)		0.212	
TIMP3 plasma /TIMP3 cell bound ratio	5.993 ± 9.051 (n=27)	2.177 ± 2.142 (n=18)		0.028	
TNFR2/TNFR1 cell bound ratio	1.127 ± 0.376 (n=23)	1.494 ± 0.604 (n=15)		0.044	
TIMP3/TACE cell bound ratio	3.927 ± 3.064 (n=28)	9.660 ± 5.239 (n=18)		<0.0001	
TNFR1 plasma /TNFR1 cell bound ratio	7.131 ± 5.796 (n=23)	7.138 ± 3.370 (n=15)		0.391	

TNFR2 plasma /TNFR2 cell bound ratio	14.57 ± 9.16 (n=23)	11.78 ± 6.58 (n=15)	0.316

ANCOVA analysis was used to compare the cohorts. Two p values were calculated to assess the impact of the covariates on the analysis. (VHR: very high risk; MR: moderate risk; LR: low risk; non-VHR: non-very high risk; SCORE: Systematic COronary Risk Evaluation risk score; TNFR1: tumour necrosis factor receptor 1; TNFR2: tumour necrosis factor receptor 2; TIMP3: tissue inhibitor of metalloproteinase 3; TACE: tumour necrosis factor alpha converting enzyme).

**adjusted for age, gender and BMI. For statistical analysis TNFR1 and TNFR2 plasma levels values were transformed using an inverse function and TIMP3 plasma levels were transformed using a log transformation.*

4.3.2 TNFR1 plasma protein levels were higher in VHR participants

To explore whether TNFR1 levels are associated with cardiovascular risk, TNFR1 plasma levels were measured in this plasma. This showed that TNFR1 plasma protein levels were significantly higher in the VHR compared to the non-VHR group (1291 ± 1158 pg/ml *vs*. 817.4 ± 208.6 pg/ml; $p<0.01$) (Figure 1a). Additionally, TNFR1 plasma levels increased significantly with increasing cardiovascular risk with the highest levels in the VHR category and the lowest levels in the LR category ($p<0.05$) (Figure 1b and Table 1).

The potential of TNFR1 plasma levels in further differentiating between VHR subgroups was examined. In terms of acute events, TNFR1 plasma levels were significantly higher in ACS-VHR participants admitted for an acute coronary syndrome compared to ELEC-VHR participants ($p<0.01$) (Figure 1c). Subsequently, TNFR1 plasma levels were investigated in order to evaluate whether they could specifically identify VHR individuals with first or recurrent acute cardiovascular events. First, data showed that TNFR1 plasma levels were numerically higher in ACS-VHR participants admitted for their first cardiovascular event who had no previous

cardiac events compared to ELEC-VHR participants with no previous cardiac events (1215.22 ± 674.81 pg/ml vs. 1052.35 ± 648.25 pg/ml; p=0.060). Second, results demonstrated that TNFR1 plasma levels were also numerically higher in ACS-VHR participants admitted for recurrent MACE (1882.92 ± 2190.94 pg/ml vs. 1196.52 ± 672.11 pg/ml; p=0.064) (Figure 1d) compared to ACS-VHR participants admitted for their first cardiovascular event.

To investigate whether TNFR1 plasma levels were associated with MACE within one year of follow-up, MACE were recorded for the recruited participants within a year of initial admission. Results showed that TNFR1 plasma levels were numerically higher on admission in participants who developed MACE after 6 months (p=0.061).

When it comes to revascularisation strategies, TNFR1 plasma levels were numerically higher in VHR participants with a previous myocardial infarction (MI) compared to VHR participants with no previous MI (1539.44 ± 1770.45 pg/ml vs. 1175.00 ± 699.17 pg/ml; p=0.069). To investigate whether this difference was independent of acute events, TNFR1 plasma levels were compared across ACS-VHR and ELEC-VHR with and without a previous MI. Results indicated that TNFR1 plasma levels were significantly higher in ACS-VHR participants with a previous MI compared to ACS-VHR participants with no previous MI (2011.18 ± 2610.37 pg/ml vs. 1262.99 ± 729.27; p<0.05) and in ELEC-VHR participants with a previous MI compared to ELEC-VHR participants with no previous MI (1210.32 ± 631.38 pg/ml vs. 1030.31 ± 592.52 pg/ml; p<0.05).

In regards to VHR participants comorbidities, TNFR1 plasma levels were significantly higher in VHR participants with diabetes compared to non-diabetics

(1537.01 ± 891.82 pg/ml vs. 1232.70 ± 1207.32 pg/ml; $p<0.001$). VHR participants with a diagnosis of heart failure had significantly higher TNFR1 plasma levels compared to VHR participants with no heart failure (2067.63 ± 2526.31 pg/ml vs. 1146.45 ± 542.96 pg/ml; $p<0.0001$) and in support of this, participants who were on diuretic therapy also had significantly higher TNFR1 plasma levels (1656.99 ± 1021.68 pg/ml vs. 1175.45 ± 1183.46 pg/ml; $p<0.0001$). A glomerular filtration rate (GFR) of less than 60 ml/min in VHR participants was associated with higher TNFR1 plasma levels (2243.31 ± 2235.13 pg/ml vs. 1066.24 ± 439.42 pg/ml; $p<0.0001$) and these participants were also more likely to be ACS-VHR individuals ($p<0.05$). TNFR1 plasma levels were also higher in VHR participants with present or previous cancer (2239.29 ± 2770.95 pg/ml vs. 1164.44 ± 625.13 pg/ml; $p<0.001$). However, TNFR1 plasma levels were lower in VHR participants with depression compared to VHR participants without depression (1195.45 ± 477.64 pg/ml vs. 1313.30 ± 1264.12 pg/ml; $p<0.05$).

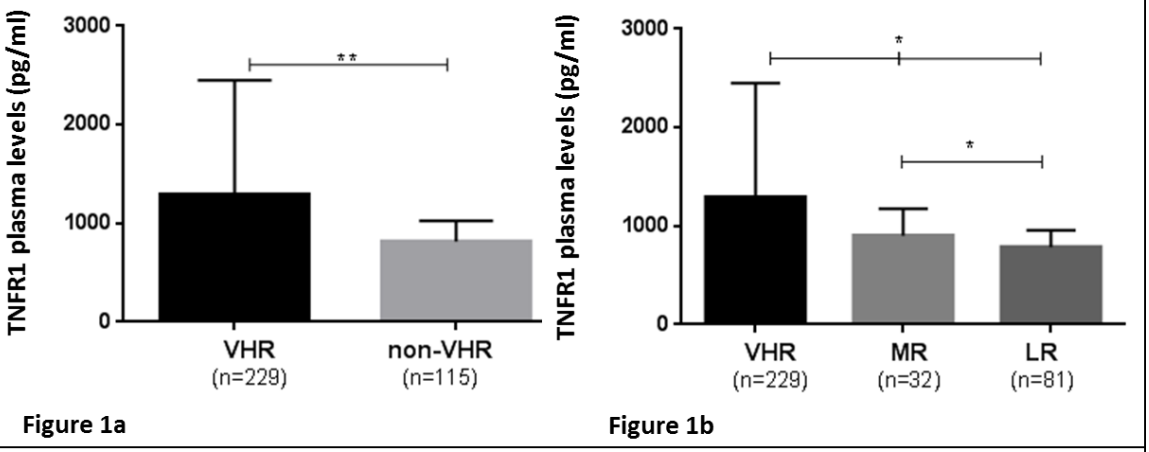


Figure 1a

Figure 1b

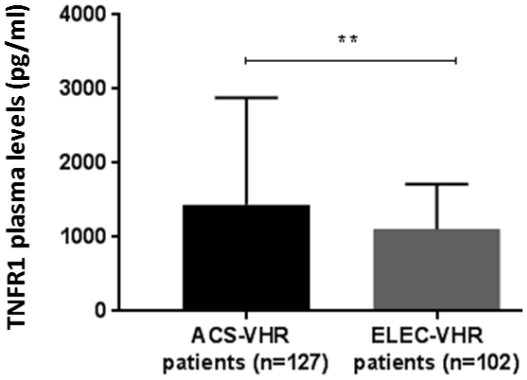


Figure 1c

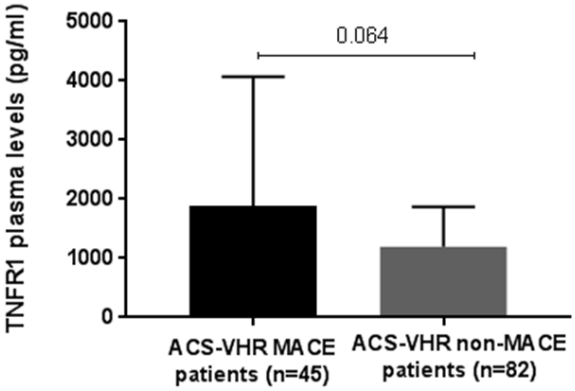


Figure 1d

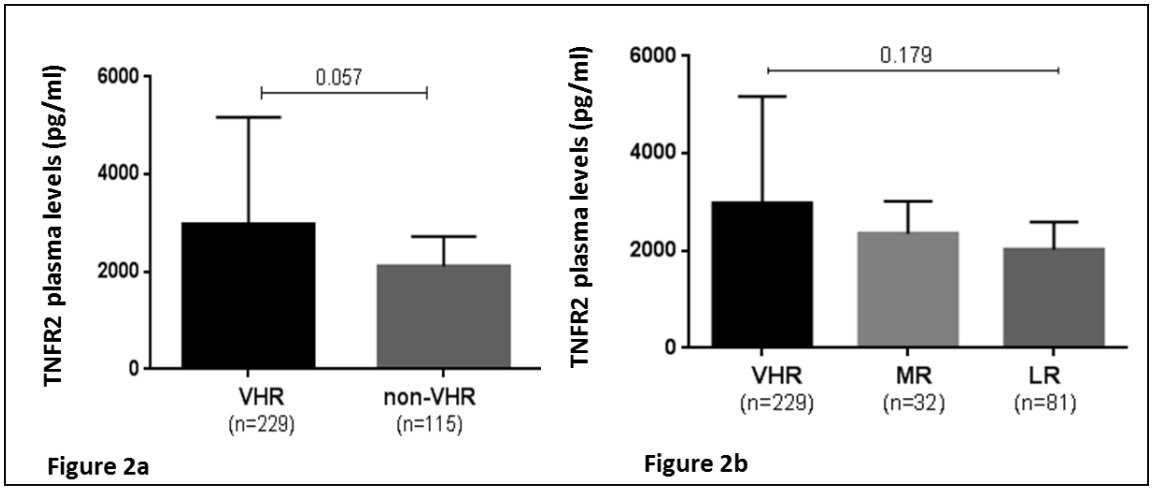
<u>Figure 1:</u> TNFR1 plasma protein levels are higher in participants at very high cardiovascular risk. *TNFR1 plasma protein levels were measured by ELISA. Figure 1a: TNFR1 plasma levels are higher VHR compared to non-VHR participants. Figure 1b: TNFR1 plasma levels are higher with increasing cardiovascular risks. Figure 1c: TNFR1 plasma levels are higher in acute coronary syndrome VHR compared to elective VHR participants, Figure 1d: TNFR1 plasma levels are higher in acute coronary syndrome VHR participants with recurrent MACE compared to acute coronary syndrome VHR participants with no recurrent MACE. (ACS: acute coronary syndrome; LR: low risk; MACE: Major adverse cardiovascular events; MI: myocardial infarction; MR: moderate risk; non-VHR: non-very high risk* participants*; PCI: Percutaneous coronary intervention; TNFα: tumour necrosis factor alpha; VHR: non-very high risk; VHR: very high risk). Statistical analysis was performed by ANCOVA for figure 1a, 1b and 1d and by Student t-test for figure 1c. **p<0.01; ****p<0.0001*p<0.05, **p<0.01*

4.3.3 TNFR2 plasma protein levels were higher in VHR participants

In order to examine whether TNFR2 was able to different between individuals at various levels of cardiovascular risk, TNFR2 levels were measured in the plasma. The current study showed that TNFR2 plasma protein levels were numerically higher in the VHR group compared to the non-VHR group but failed to reach statistical significance (2971.0 ± 2199.0 pg/ml vs. 2133.0 ± 614.0 pg/ml; p=0.057) (Figure 2a, Figure 2b and Table 1). However, there was no statistical difference in TNFR2 plasma levels between ACS-VHR and ELEC-VHR participants (p=0.185) or in VHR participants with a previous MI (p=0.530) or PCI (p=0.373). Additionally, TNFR2 plasma levels were not associated with MACE within one year of admission (p=0.453).

Regarding the comorbidities present in VHR participants, similarly to TNFR1, TNFR2 plasma levels were significantly higher in VHR participants with diabetes (3269.52 ± 1478.42 pg/ml vs. 2899.71 ± 2335.73 pg/ml; p<0.05) compared to VHR participants with no diabetes. TNFR2, like TNFR1, plasma levels discriminated between VHR participants with heart failure compared to VHR participants with no heart failure (4066.22 ± 4562.90 pg/ml vs. 2766.43 ± 1296.48 pg/ml; p<0.01). Furthermore, like TNFR1, TNFR2 plasma levels were higher in VHR participants on diuretic therapy compared to VHR participants not on diuretic therapy (3363.79 ± 1474.43 pg/ml vs. 2835.63 ± 2380.94 pg/ml; p<0.05). VHR participants with a GFR less than 60 ml/min also had higher TNFR2 plasma levels (4546.22 ± 4095.44 pg/ml vs. 2606.99 ± 1112.57 pg/ml; p<0.0001) (Table 2).

To explore whether TNFR1 and TNFR2 were collectively associated with cardiovascular risk, ratio of TNFR1 to TNFR2 plasma protein levels was calculated. Results indicated that ratio of TNFR1 to TNFR2 plasma protein levels was not significantly different between the VHR and the non-VHR group (p=393). Interestingly, ratio of TNFR1 to TNFR2 plasma protein levels was higher in ACS-VHR compared to ELEC-VHR participants (0.4701 ± 0.2329 vs. 0.4157 ± 0.1025; p<0.05). R1/R2 ratio was significantly higher in VHR participants with heart failure compared to VHR participants with no heart failure (0.5192 ± 0.3172 vs. 0.4322 ± 0.1498; p<0.05 respectively). Nevertheless, R1/R2 ratio was not significantly different between the VHR and the non-VHR group (*p=0.393*).



<u>Figure 2:</u> TNFR2 plasma protein levels in participants at various levels of cardiovascular risk. *TNFR2 plasma protein levels were measured by ELISA. Figure 2a: TNFR2 plasma levels in VHR compared to non-VHR participants. Figure 2b: TNFR2 plasma levels across VHR, MR and LR participants. (LR: low risk; MR: moderate risk; non-VHR: non-very high risk; TNFR2: tumour necrosis factor alpha receptor 2; VHR: very high risk). Statistical analysis was performed by ANCOVA.*

4.3.4 TIMP3 plasma protein levels were not statistically different between VHR and non-VHR participants whereas *TIMP3* mRNA levels were lower in VHR participants

To investigate if TIMP3 levels could differentiate between individuals at various levels of cardiovascular risk, TIMP3 protein and gene expression levels were measured. Results showed that TIMP3 plasma levels were not statistically different between VHR and non-VHR participants ($p=0.486$) (Figure 3a and Table 1). However, TIMP3 plasma levels were higher in VHR participants on antihypertensive therapy and anti-anginal therapy (3679.15 ± 2933.52 pg/ml vs. 2507.24 ± 1218.44 pg/ml; $p<0.01$ and 3737.67 ± 2820.73 pg/ml vs. 3184.70 ± 2554.98 pg/ml; $p<0.05$ respectively). When it comes to *TIMP3* gene expression, results demonstrated that *TIMP3* mRNA levels were lower in VHR compared to no-VHR participants (0.0001094 ± 0.0001122 vs. 0.0001946 ± 0.000305; $p<0.05$) (Figure 3b). To investigate whether *TIMP3* gene expression was different in VHR subgroups, ACS-VHR and ELEC-VHR participants were compared. Data showed that *TIMP3* gene expression was lower in ACS-VHR compared to ELEC-VHR participants (0.00007818 ± 0.00005755 (n=15) vs. 0.0001595 ± 0.0001562 (n=24); $p<0.05$).

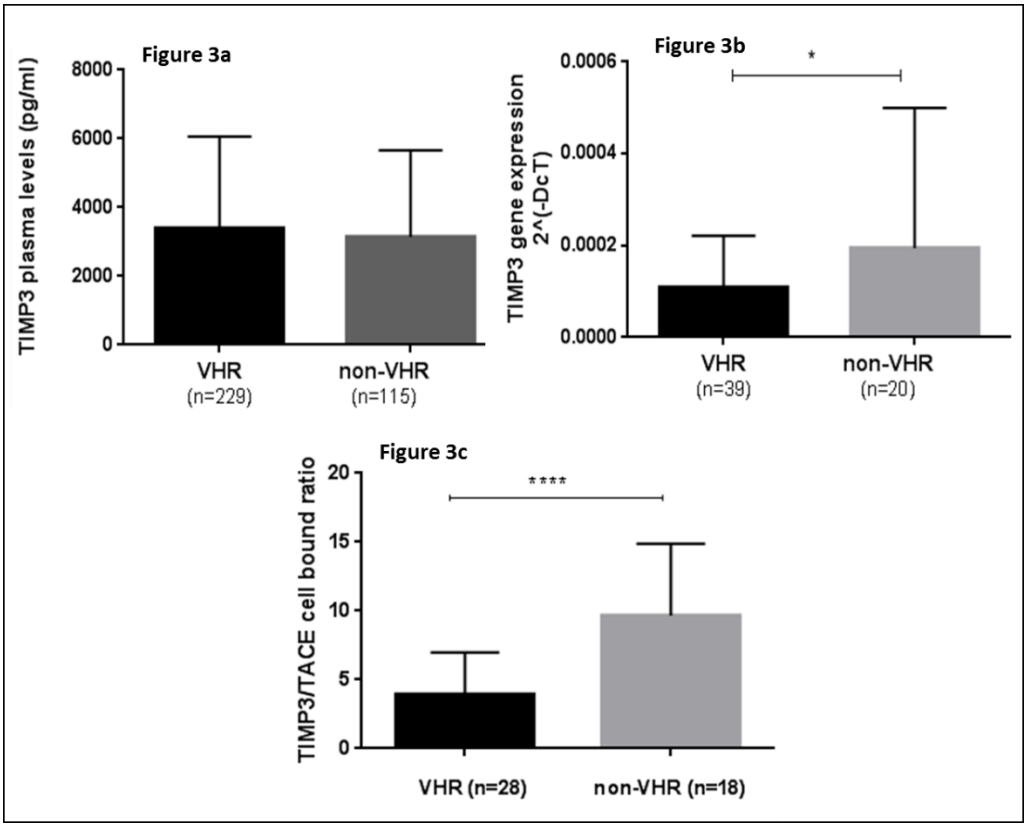


Figure 3: ***TIMP3 levels in VHR and non VHR participants.*** *TIMP3 plasma protein levels were measured by ELISA. TIMP3 gene expression was measured was measured in the peripheral blood mononuclear cells (PBMCs) by quantitative PCR and normalised to GAPDH. Cell membrane bound TACE and TIMP3 levels were measured by ELISA following a total protein extraction from the PBMCs after normalisation to 5000ug/m of total protein. Figure 3a: TIMP3 plasma levels in VHR* participants *compared to non-VHR participants. Figure 3b: TIMP3 gene expression is lower in VHR compared to non VHR participants. Figure 3c: Cell bound TIMP3 over TACE ratio is lower in VHR compared to non-VHR participants (non-VHR: non-very high risk; TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: tissue inhibitor of metalloproteinase 3; VHR: very high risk). Statistical analysis was performed by ANCOVA for figure 3a and Student t-test for the rest. *p<0.05, ****p<0.0001*

4.3.5 Cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels in VHR participants

To further validate the results of plasma TNFR1, TNFR2 and TIMP3 protein levels, cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels were explored across individuals at various levels of cardiovascular risk. Cell membrane bound

TNFR1, TNFR2, TIMP3 and TACE protein levels were measured in a subgroup of participants randomly selected. Results demonstrated that cell membrane bound TACE protein levels were higher in VHR (n=80) vs. non VHR participants (n=36) (383.9 pg/ml ± 259.6 vs. 221.9 pg/ml ± 78.8; $p<0.0001$).

In addition, cell membrane bound TNFR1 protein levels were higher in VHR vs. non VHR participants ($p<0.01$) (Figure 4a) however, cell membrane bound TNFR2 levels were not significantly different ($p=0.212$). Interestingly, the ratio of cell membrane bound TNFR2 to TNFR1 protein levels was lower in VHR participants compared to non-VHR participants (1.127 ± 0.376 vs. 1.494 ± 0.604; $p<0.05$) (Figure 4c).

On the other hand, the ratio of plasma TIMP3 over cell membrane bound TIMP3 protein levels was higher in VHR compared to non VHR participants (5.993 ± 9.051 vs. 2.177 ± 2.142; $p<0.05$). Nevertheless, the ratio of cell membrane bound TIMP3 to TACE protein levels was lower in VHR compared to non VHR participants (3.927 ± 3.064 vs. 9.660 ± 5.239; $p<0.0001$) (Figure 3c).

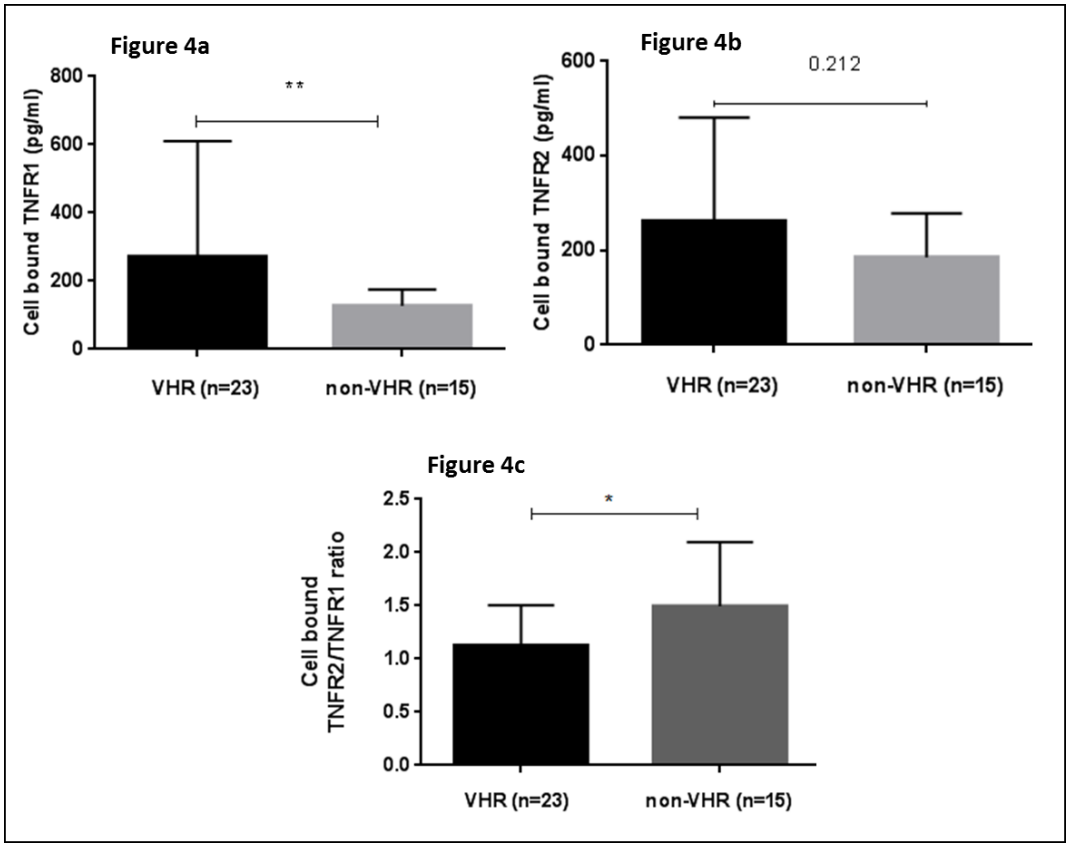


Figure 4: ***Cell membrane bound TNFR1 and TNFR2 protein levels in VHR and non VHR participants.*** *Cell membrane bound TNFR1 and TNFR2 protein levels were measured by ELISA following a total protein extraction from the PBMCs after normalisation to 5000ug/ml. Figure 4a: Cell membrane bound TNFR1 protein levels are higher in VHR compared to non VHR participants. Figure 4b: Cell membrane bound TNFR2 protein levels in VHR compared to non VHR participants. Figure 4c: Cell membrane bound TNFR2 over TNFR1 protein levels ratio is lower in VHR compared to non VHR participants. (non-VHR: non-very high risk; TNFR1: tumour necrosis factor alpha receptor 1, TNFR2: tumour necrosis factor alpha receptor 2; VHR: very high risk). Statistical analysis was performed by Student t-test. *p<0.05*

4.3.6 TNFR1 and TNFR2 plasma protein levels correlated positively

To explore whether TNFR1 and TNFR2 levels correlated with different variables explored in the study, a correlation analysis was performed (section 3.2.7 in Chapter 3). TNFR1 plasma protein levels correlated positively with TNFR2 levels (r=0.735;

p<0.0001). In addition, TNFR1 plasma protein levels correlated positively with age, body mass index (BMI) and C reactive protein (CRP) levels (r=0.515; p<0.0001, r=0.164; p<0.01; 0.164; p<0.01) and negatively with cholesterol levels (r=-0.275; p<0.0001). On the other hand, TNFR2 plasma protein levels correlated positively with age and BMI (r=0.390; p<0.0001 and r=0.174; p<0.01) and negatively with cholesterol levels (r=-0.274; p<0.0001) (Table 2).

<u>Table 2:</u> Correlation matrix table between TACE, TNFα, CRP, age, BMI, systolic BP and total cholesterol levels.

		TNFR1 plasma levels(pg/ml)	TNFR2 plasma levels(pg/ml)	TIMP3 plasma levels (pg/ml)	Age (years)	BMI (kg/m²)	Systolic blood pressure (mmHg)	Cholesterol (mmol/L)	CRP levels (mg/ml)
TNFR1 plasma levels(pg/ml)	Pearson Correlation	1	.735**	.038	-.515**	-.164**	-.054	.275**	-.164**
	Sig. (2-tailed)		.000	.486	.000	.003	.360	.000	.005
	N	340	340	340	340	316	292	314	290
TNFR2 plasma levels(pg/ml)	Pearson Correlation	.735**	1	.065	-.390**	-.174**	-.025	.274**	-.061
	Sig. (2-tailed)	.000		.232	.000	.002	.670	.000	.300
	N	340	340	340	340	316	292	314	290
TIMP3 plasma levels (pg/ml)	Pearson Correlation	.038	.065	1	.043	-.041	.028	.011	.104
	Sig. (2-tailed)	.486	.232		.428	.464	.640	.845	.078
	N	340	340	340	340	316	292	314	290
Age (years)	Pearson Correlation	-.515**	-.390**	.043	1	.061	.212**	-.303**	.101
	Sig. (2-tailed)	.000	.000	.428		.280	.000	.000	.086
	N	340	340	340	340	316	292	314	290
BMI (kg/m2)	Pearson Correlation	-.164**	-.174**	-.041	.061	1	.103	-.012	.090
	Sig. (2-tailed)	.003	.002	.464	.280		.089	.834	.140
	N	316	316	316	316	316	275	292	268
Systolic blood pressure (mmHg)	Pearson Correlation	-.054	-.025	.028	.212**	.103	1	.076	-.112
	Sig. (2-tailed)	.360	.670	.640	.000	.089		.212	.080
	N	292	292	292	292	275	292	271	247
Cholesterol (mmol/L)	Pearson Correlation	.275**	.274**	.011	-.303**	-.012	.076	1	-.116
	Sig. (2-tailed)	.000	.000	.845	.000	.834	.212		.058
	N	314	314	314	314	292	271	314	267
CRP levels (mg/ml)	Pearson Correlation	-.164**	-.061	.104	.101	.090	-.112	-.116	1
	Sig. (2-tailed)	.005	.300	.078	.086	.140	.080	.058	
	N	290	290	290	290	268	247	267	290

Highlighted cells refer to a significant correlation. TNFR1 and TNFR2 plasma levels were transformed using an inverse function, therefore, the correlation coefficients have an inversed sign and were interpreted in opposite. (BMI: body mass index; CRP: C-reactive protein; non-VHR: non-very high risk; TACE: Tumour necrosis factor alpha converting

enzyme; TIMP3: tissue inhibitor of metalloproteinase 3; TNFα: Tumour necrosis factor alpha; TNFR1: tumour necrosis factor alpha receptor 1; TNFR2: tumour necrosis factor alpha receptor 2; VHR: very high risk). Pearson's correlation was used to evaluate the correlation between variables.

**Correlation is significant at the 0.05 level*
***Correlation is significant at the 0.01 level*

4.3.7 CRP plasma levels did not further differentiate between VHR participant sub-groups

Refer to **section 3.3.6** in **Chapter 3.**

4.3.8 TNFR1, TNFR2 and TIMP3 plasma protein levels did not predict angiographic outcome

To explore whether TNFR1, TNFR2 and TIMP3 levels could determine the plaque burden in VHR participants, a multinomial logistic regression analysis was carried out (section 3.2.7 in Chapter 3). A multinomial logistic regression analysis showed that the measured biomarkers were not able to effectively predict the angiographic outcome. In addition, ANOVA analysis did not show any difference between the biomarker levels and the different categories of angiogram outcomes.

Table 3: ***TNFR1, TNFR2 and TIMP3 plasma protein levels in very high risk participants.***

	TNFR1 plasma levels (pg/ml)	***p value***	**TNFR2 plasma levels (pg/ml)**	***p value***	**TIMP3 plasma levels (pg/ml)**	***p value***
Demographic and History *(n; %)*						
Gender						
Male *(180; 78.6)*	1273.12 ± 1246.12	0.275	2909.79 ± 2319.03	0.329	3401.05 ± 2720.93	0.897
Female *(49; 21.4)*	1357.47 ± 756.48		3194.75 ± 1688.92		3382.75 ± 2491.47	
Diabetes mellitus						
Present *(44; 19.2)*	1537.01 ± 891.82	0.001	3269.52 ± 1478.42	0.029	3636.29 ± 2630.16	0.268
Absent *(185; 80.8)*	1232.70 ± 1207.32		2899.71 ± 2335.73		3340.25 ± 2681.06	
Heart Failure						
Present *(36; 15.7)*	2067.63 ± 2526.31	<0.0001	4066.22 ± 4562.90	0.008	3673.14 ± 2809.42	0.324
Absent *(193; 84.3)*	1146.45 ± 542.96		2766.43 ± 1296.48		3345.65 ± 2645.44	
Renal function						
GFR < 60 *(45; 20)*	2243.31 ± 2235.13	<0.0001	4546.22 ± 4095.44	<0.0001	3295.58 ± 3021.49	0.721
GFR > 60 *(180; 80)*	1066.24 ± 439.42		2606.99 ± 1112.57		3440.58 ± 2600.27	
Hypertension						
Present *(128; 55.9)*	1432.94 ± 1420.85	0.102	3218.53 ± 2740.46	0.547	3492.06 ± 2716.37	0.367
Absent *(101; 44.1)*	1111.50 ± 659.34		2656.76 ± 1144.93		3276.83 ± 2614.35	
Dyslipidaemia						
Present *(134; 58.5)*	1166.50 ± 600.69	0.249	2734.97 ± 1196.20	0.285	3426.48 ± 2439.58	0.368
Absent *(95; 41.5)*	1467.03 ± 1639.55		3303.36 ± 3084.65		3355.74 ± 2973.91	
Arthritis						
Present *(35; 15.3)*	1269.50 ± 721.23	0.64	2781.90 ± 1099.22	0.958	3735.10 ± 2699.06	0.337
Absent *(194; 84.7)*	1295.09 ± 1221.55		3004.84 ± 2343.69		3336.15 ± 2665.05	
Depression						
Present *(43; 18.8)*	1195.45 ± 477.64	0.028	2729.37 ± 1044.05	0.336	3692.24 ± 3211.30	0.653
Absent *(186; 81.2)*	1313.30 ± 1264.12		3026.57 ± 2386.70		3328.91 ± 2531.38	
present or previous cancer						
Present *(27; 11.8)*	2239.29 ± 2770.95	0.001	4502.74 ± 5201.40	0.075	3255.11 ± 1956.31	0.666
Absent *(202; 88.2)*	1164.44 ± 625.13		2765.99 ± 1276.33		3416.12 ± 3752.71	
Smoking						
none *(66; 30.3)*	1157.64 ± 531.68	0.074	2899.58 ± 1333.70	0.896	3320.69 ± 2627.36	0.667
ex-smoker *(110; 50.4)*	1430.37 ± 1572.08		3124.24 ± 2837.42		3540.49 ± 2798.60	
current *(42; 19.3)*	1118.28 ± 307.86		2539.00 ± 827.09		3293.09 ± 2683.45	
Pharmacotherapy *(n; %)*						
Statin therapy						
Present *(165; 72.7)*	1331.99 ± 1324.71	0.733	3028.38 ± 2463.10	0.861	3527.96 ± 2851.86	0.292
Absent *(62; 27.3)*	1193.78 ± 521.57		2799.71 ± 1265.70		3041.89 ± 2082.24	
Antiplatelet therapy						
Present *(154; 67.8)*	1325.28 ± 1329.44	0.154	2952.38 ± 2393.44	0.369	3635.63 ± 3006.40	0.171
Absent *(73; 32.1)*	1228.78 ± 690.99		2994.51 ± 1742.01		2888.00 ± 1659.95	
Antihypertensive therapy						
Present *(172; 76.8)*	1347.68 ± 1302.31	0.683	2986.69 ± 2352.67	0.445	3679.15 ± 2933.52	0.006
Absent *(55; 24.2)*	1127.14 ± 494.53		2900.98 ± 1656.87		2507.24 ± 1218.44	
Anti-anginal therapy						
Present *(88; 38.8)*	1371.57 ± 1614.24	0.976	3093.82 ± 3039.83	0.848	3737.67 ± 2820.73	0.037

Absent *(139; 61.2)*	1245.30 ± 750.31		2885.00 ± 1446.55		3184.70 ± 2554.98	
Diuretic therapy						
Present *(56; 24.7)*	1656.99 ± 1021.68	<0.0001	3363.79 ± 1474.43	0.013	3475.25 ± 2424.43	0.404
Absent *(171; 75.3)*	1175.45 ± 1183.46		2835.63 ± 2380.94		3368.99 ± 2749.54	
Drug naïve						
Present *(54; 23.7)*	1095.19 ± 477.23	0.782	2826.33 ± 1640.02	0.813	2760.77 ± 1363.49	0.147
Absent *(174; 76.3)*	1352.19 ± 1296.73		3001.65 ± 2354.62		3578.09 ± 2933.57	
Clinical Variables *(n; %)*						
ACS-VHR *(127; 55.5)*	1439.73 ± 1440.60	0.002	3198.97 ± 2762.81	0.185	3356.92 ± 2527.43	0.988
ELEC-VHR *(102; 44.5)*	1106.60 ± 612.67		2786.74 ± 1117.28		3447.20 ± 2845.62	
Previous MI						
Present *(73; 31.9)*	1539.44 ± 1770.45	0.069	3397.89 ± 3388.02	0.53	3385.49 ± 2856.55	0.965
Absent *(156; 68.1)*	1175.00 ± 699.17		2770.90 ± 1286.84		3402.60 ± 2584.89	
Previous MI ACS						
Present (30; 23.6)	2011.18 ± 2610.37	0.035	4261.47 ± 5012.56	0.398	3013.39 ± 1608.56	0.79
Absent (97; 76.4)	1262.99 ± 729.27		2870.23 ± 1402.28		3463.15 ± 2748.49	
Previous MI ELEC						
Present (43; 42.1)	1210.32 ± 631.38	0.036	2795.39 ± 1193.57	0.583	3645.09 ± 3468.99	0.761
Absent (59; 57.8)	1030.31 ± 592.52		2607.56 ± 1061.62		3302.98 ± 2310.09	
Previous PCI						
Present *(73; 31.9)*	1476.43 ± 1758.32	0.482	3320.81 ± 3299.32	0.373	3341.96 ± 2772.40	0.989
Absent *(56; 68.1)*	1204.50 ± 715.78		2807.00 ± 1404.60		3423.00 ± 2626.73	
Previous PCI ACS						
Present (28; 22.0)	1969.82 ± 2695.94	0.076	4198.36 ± 5084.83	0.133	3184.46 ± 1734.11	0.737
Absent (99; 78.0)	1289.81 ± 750.23		2916.19 ± 1524.59		3405.69 ± 2715.44	
Previous PCI ELEC						
Present (45; 44.1)	1169.43 ± 589.81	0.134	2774.78 ± 1073.21	0.305	3439.96 ± 3272.09	0.744
Absent (57; 55.9)	1056.28 ± 630.80		2617.24 ± 1155.56		3452.92 ± 2488.40	
Previous CABG						
Present *(22; 9.6)*	1261.80 ± 790.64	0.225	2680.20 ± 939.42	0.287	3339.40 ± 2147.24	0.884
Absent *(207; 90.4)*	1294.29 ± 1191.69		3001.65 ± 2291.92		3403.27 ± 2721.97	
Previous MI, PCI or stent						
Present *(100; 43.7)*	1462.44 ± 1568.07	0.399	3248.79 ± 2937.52	0.375	3433.87 ± 2665.03	0.575
Absent *(129; 56.3)*	1158.41 ± 667.68		2755.24 ± 1354.08		3368.65 ± 2680.78	
Diagnosis upon admission						
Stable Angina (85; 37.1)	1089.76 ± 607.54	<0.0001	2665.46 ± 1184.38	0.324	3504.16 ± 3244.50	0.994
Unstable Angina (21; 9.2)	1217.10 ± 718.27		2885.19 ± 1565.10		3058.00 ± 1763.03	
NSTEMI < 1 week (77; 33.6)	1599.52 ± 1773.96		3417.92 ± 3382.58		3330.37 ± 2372.66	
STEMI < 1 week (7; 3.0)	1240.86 ± 374.45		2763.00 ± 695.33		3080.14 ± 1336.24	
Other (39; 17.03)						
ACS participants admitted for MACE						
Present *(45; 35.4)*	1882.92 ± 2190.94	0.064	3877.78 ± 4147.86	0.267	3105.04 ± 1775.69	0.902
Absent *(82; 64.6)*	1196.52 ± 672.11		2826.30 ± 1459.91		3495.15 ± 2858.20	
No previous MI, PCI or CABG						

ACS-VHR *(84, 65.1)*	1215.22 ± 674.81	0.06	2855.92 ± 1455.10	0.316	3455.36 ± 2836.15	0.725
ELEC-VHR *(45, 34.9)*	1052.35 ± 648.25		2567.31 ± 1133.34		3206.81 ± 2385.44	
Prognostic variable *(n; %)*						
Follow-up MACE						
Within 6 months *(5; 2.2)*	1708.04 ± 930.91	0.061	3847.00 ± 1939.68	0.453	3842.40 ± 3333.99	0.624
After 6 months *(11; 4.8)*	2137.97 ± 1618.90		3379.18 ± 1928.64		2741.85 ± 1579.92	
Absent *(213; 93.0)*	1237.65 ± 1121.52		2929.10 ± 2219.55		3420.52 ± 2702.14	

Cells highlighted with dark grey have a p value <0.05 and cells highlighted with light grey have a p value of <0.01. GFR: Glomerular filtration rate; ACS: acute coronary syndrome participants*; ELEC: elective* participants*; MI: Myocardial Infarction, PCI: percutaneous coronary intervention CABG: Coronary artery bypass surgery; NSTEMI: non-ST elevation myocardial infarction; STEMI: ST elevation myocardial infarction; MACE: major adverse cardiovascular events*

4.4 Discussion

The results of this present investigation demonstrated that TNFR1, TNFR2 and TIMP3 could identify individuals at cardiovascular risk and could also further stratify those at higher risk of MACE demonstrating a higher value compared to CRP measurement. Cell membrane bound TNFR1, TNFR2, TIMP3 and TACE protein levels provide further insight on the potential role of this inflammatory pathway as a therapeutical target in CVD.

4.4.1 TNFR1 and TNFR2 plasma protein levels characterised VHR individuals

Soluble TNFR1 and TNFR2 circulating levels have been previously linked to different inflammatory diseases (206) and were previously explored in CVD (207). However, in the aforementioned studies, soluble TNFR1 and TNFR2 levels were only associated with the presence of co-morbidities in CVD participants and were not compared across individuals at various levels of cardiovascular risk.

The present investigation demonstrated that TNFR1 plasma protein levels were higher in VHR compared to non-VHR participants. Furthermore, increasing levels of TNFR1 plasma levels accompanied increasing cardiovascular risk in parallel to the assigned SCORE risk. In addition, TNFR2 plasma protein levels followed a trend where levels were higher in VHR compared to non-VHR participants. This suggests that TNFR1 and TNFR2 plasma levels could both be considered as markers for cardiovascular risk with TNFR1 proving to be of higher sensitivity in characterising cardiovascular risk compared to TNFR2. Furthermore, TNFR1 plasma protein levels tended to be higher in VHR participants admitted for a primary or recurrent cardiovascular event which shows that TNFR1 could also be considered as a marker

for acute events in individuals with or without CVD history. This difference was not detected by CRP levels which suggests that TNFR1 has a superior value compared to CRP and could potentially be useful in further stratifying VHR individuals.

When it comes to individuals with a previous MI, our results demonstrated that TNFR1 plasma protein levels remained high in VHR participants with a previous MI despite statin, anti-platelet and anti-hypertensive therapy. This was also observed when we looked at ACS-VHR and ELEC-VHR participants with a previous MI. Nonetheless, CRP levels were not statistically different between VHR participants with previous MI compared to VHR participants with no previous MI which further strengthens the role of TNFR1 as a potential biomarker in further stratifying VHR individuals. On the other hand, based on the evidence that both soluble levels of TNFR1 and TNFR2 are known to contribute to inflammation (207) the ratio of TNFR1 and TNFR2 plasma levels was explored. Higher TNFR1 to TNFR2 plasma ratio in ACS-VHR compared to ELEC-VHR participants reflected the acute phase response following an ACS and the possibility of using such a ratio in ACS diagnosis.

Moreover, TNFR1 plasma protein levels appeared to be higher in VHR participants who developed MACE after one year of admission which is in line with previous findings highlighting the role of TNFR1 in predicting mortality in patients post-MI (222). This supports the role of TNFR1 not only as a diagnostic marker for acute MACE but also as a prognostic marker for future MACE. These results need to be replicated in larger cohorts.

On the other hand, TNFR1 and TNFR2 plasma protein levels were higher in VHR participants with diabetes and heart failure indicating that levels tend to be higher

when certain comorbidities are present along with CVD as reported previously (207,223). In fact, since it has been shown that TNFR1 and TNFR2 plasma protein levels were higher in diabetic patients who developed CVD as a complication, this suggests that these two soluble receptors could be associated with the development of cardiovascular complications in an already established disease such as diabetes (206) or chronic kidney disease (224).

4.4.2 TNFR1 and TNFR2 cell membrane bound protein levels in VHR individuals

It is well established that TNFR1 and TNFR2 receptors have opposing actions when it comes to their signalling pathways (208). TNFR1 increases inflammation and endothelial dysfunction whereas TNFR2 activates angiogenic and survival pathways (209,210). The opposing roles of TNFR1 and TNFR2 cell membrane receptors has also been highlighted in neurodegenerative disease (225), where cell membrane bound TNFR1 to TNFR2 receptor ratio was increased in the hippocampus with aging (226), and also in heart failure (227). In this present work, cell membrane bound TNFR2 to TNFR1 receptor ratio was explored in the peripheral blood cells to provide further insight into its role in CVD. TNFR2 to TNFR1 receptor ratio was lower in VHR compared to non-VHR participants. This indicates that VHR participants have a low number of TNFR2 relative to TNFR1 on the cell surface of their peripheral blood cells which could suggest that the balance between those receptors is more likely to shift towards a pro-inflammatory state potentially increasing MACE risk in the VHR group. In line with these findings, cell membrane bound TNFR1 protein levels were higher in VHR compared to non-VHR participants. It has been reported that TNFR2 to TNFR1

receptor ratio is important in modulating cell response to TNFα (228). When it comes to the regulation of TNF receptors, it has been suggested that the TNFR1 promoter is constitutively active at low levels in all cell types (229) whereas TNFR2 promoter is inducible and expressed exclusively by immune cells, endothelial cells and some neuronal populations (230). This indicates that the presence of TNFR2 on those cell types is essential for modulating the inflammatory response to TNFα.

4.4.3 *TIMP3* is downregulated in VHR individuals

TIMP3 is the only known endogenous inhibitor of TACE (217). In this present study, TIMP3 plasma protein levels were not statistically different between participants at various levels of cardiovascular risk. However, since TIMP3 is known to regulate blood pressure (231), plasma levels were found to be higher in VHR participants on antihypertensive therapy compared to VHR participants not on antihypertensive therapy which indicates a need to further understand the potential benefit of higher TIMP3 levels as a result of anti-hypertensive therapy. Nevertheless, our results showed that *TIMP3* was under expressed in VHR compared to non-VHR participants. This suggests that, in contrast to an increase in *TACE* gene expression in patients with CVD (170), *TIMP3* gene expression is decreased. *TIMP3* was also downregulated in ACS-VHR participants compared to ELEC-VHR participants indicating that close to an ACS, *TIMP3* gene expression is particularly decreased. Down-regulation of *TIMP3* has been previously shown to increase *TACE* expression and TNFα production by placental trophoblast cells (232). In addition, a loss of *TIMP3* has shown to increase the risk of atherosclerosis in ApoE null mice (233). However, the mechanisms behind *TIMP3* downregulation in CVD need further investigation especially in a context

where TIMP3 is showing utility as a potential therapeutical strategy in heart failure (234).

The ratio of plasma TIMP3 over cell bound TIMP3 protein levels was higher in VHR compared to non VHR individuals indicating that in VHR individuals, a higher proportion of TIMP3 is released into the plasma compared to the amount of TIMP3 retained on the cell surface. TIMP3 retained on the cell surface is known to be associated with TACE to form dimer structures that inhibit TACE activity and substrate shedding (235,236). A lower proportion of TIMP3 on the cell surface results in an insufficient TACE inhibition which was reflected by the cell membrane bound TIMP3 to TACE protein levels ratio which was significantly lower in VHR participants. When TACE is ineffectively inhibited by TIMP3, it shifts into its monomer active form, and subsequently cleaves a higher number of transmembrane proteins aggravating local and systemic inflammation (235). TIMP3 has been recently suggested to decrease adipocyte differentiation (237) which highlights a potential role of this protein in the lipid retention and possibly in the formation of an atherosclerotic lesion (238).

4.4.4 Strengths and Limitations

Refer to **section 3.4.3 in Chapter 3**.

In addition to the aforementioned limitations, the number of participants tested for membrane bound forms of TNFR1, TNFR2 and TIMP3 was limited, however, preliminary data shows a significant difference between the levels of those surface markers as well as the ratios between VHR and non VHR participants and ongoing research is investigating this association in a larger number of participants.

Concerning the inter-assay CV of the TIMP3 ELISA, the reasons behind a high CV are discussed in the Methods section II.IX.I. This shows that TIMP3 measurement over time in the sample plasma sample resulted in a high inter-assay CV which would suggest that TIMP3 might degrade over time and might need to be measured as close to blood collection as possible.

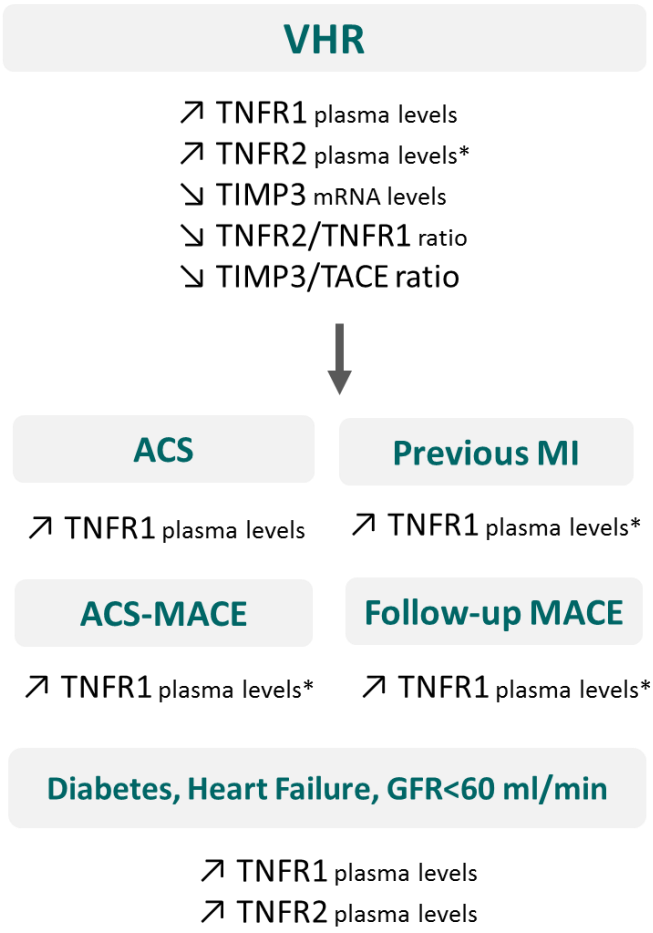


***Figure 5:* Proposed model using TNFR1, TNFR2 and TIMP3 in identifying and further stratifying VHR participants**. *(ACS: acute coronary syndrome* participants*; GFR: Glomerular filtration rate; MACE: major adverse cardiovascular events MI: Myocardial Infarction; PCI: percutaneous coronary intervention; TNFR1: tumour necrosis factor receptor 1; TNFR2: tumour necrosis factor receptor 2; TIMP3: tissue inhibitor of metalloproteinase 3; TACE: tumour necrosis factor alpha converting enzyme; VHR: very high risk). *Refers to p values that nearly reached significance.*

4.5 Conclusion

These results indicate that plasma TNFR1 and TNFR2 protein levels can differentiate between VHR and non-VHR participants. Additionally, TNFR1 can identify individuals admitted for their first or secondary acute cardiovascular event. This is the first report of *TIMP3* downregulation in CVD individuals. Furthermore, the ratio of cell membrane bound TNFR2 to TNFR1 and TIMP3 to TACE protein levels were also investigated for the first time and were both lower in VHR compared to non-VHR participants highlighting a shift towards a pro-inflammatory state in the VHR group. These proteins should be further investigated as biomarkers or therapeutic targets in CVD.

Chapter 5

Evaluating the Value of a Proinflammatory Panel in Cardiovascular Risk Assessment

Abstract

Background: The immune system plays a major role in the initiation and progression of atherosclerosis which results in coronary artery disease (CAD), one of the major subtypes of cardiovascular disease (CVD). Several treatment strategies targeting the immune system are currently being developed to decrease CAD occurrence and complications. A large number of inflammatory mediators are known to contribute to atherosclerosis, however, their role in predicting cardiovascular risk and recurrent cardiac events is not fully characterised. The aim of this present work was to evaluate the value of a panel combining several inflammatory proteins in determining cardiovascular risk and in further stratifying participants with an established CAD.

Methods: Participants were recruited from the cardiac catheterisation laboratory or by email advertisement. Group 1 was defined as very high risk participants (VHR) with a 10-year risk SCORE ≥10% risk of fatal CVD. VHR participants were subdivided in Acute Coronary Syndrome (ACS-VHR) and elective percutaneous coronary intervention participants (ELEC-VHR). Group 2 were defined as low, moderate and high risk participants (non-VHR) with a 10-year risk SCORE <10% risk of fatal CVD. INF-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNFα plasma protein levels were measured using the MSD® MULTI-SPOT Assay System.

Results: At total of 344 participants were recruited. The VHR (n=229) group consisted of ACS-VHR (n=127) and ELEC-VHR (n=102). The non-VHR (n=115) group consisted of low risk (LR) (n=81), moderate risk (MR) (n=32) and high risk (HR) (n=2) participants. IFN-γ (9.93 ± 42.02 pg/ml vs. 8.22 ± 11.43 pg/ml, p<0.01), IL-6 (2.06 ± 2.80 pg/ml vs. 0.066 ± 0.512 pg/ml, p<0.0001), IL-8 (8.16 ± 10.00 pg/ml vs. 3.50 ± 1.99 pg/ml, p<0.0001) and TNFα (4.711 ± 2.453 pg/ml *vs.* 2.770 ± 0.752 pg/ml, p<0.0001) plasma

protein levels were able to significantly differentiate between very high risk (VHR) and non-VHR participants. *IL1B* mRNA levels were higher in the VHR compared to non-VHR participants (0.0654 ± 0.0413 vs. 0.0335 ± 0.0272; $p<0.01$). IFN-γ (10.19 ± 53.03 pg/ml vs. 8.52 ± 9.12 pg/ml; $p<0.01$) and IL-6 (2.75 ± 3.57 pg/ml vs. 1.20 ± 0.70 pg/ml; $p<0.001$) plasma levels were higher in ACS-VHR compared to ELEC-VHR. IFN-γ (20.88 ± 89.61 pg/ml vs. 4.52 ± 3.78 pg/ml; $p<0.01$), IL-12p70 (0.35 ± 0.44 pg/ml vs. 0.28 ± 0.42 pg/ml; $p<0.05$) and IL-8 (9.87 ± 10.92 pg/ml vs. 7.48 ± 12.31 pg/ml; $p<0.01$) plasma levels were higher in ACS participants admitted for recurrent major adverse cardiovascular events (MACE). IL-6 plasma levels were associated with follow up MACE within 6 months of admission ($p<0.05$).

Conclusion: This study shows that IL-6, IL-8, INF-γ, TNFα and IL-1β can differentiate between VHR and non-VHR CVD participants. Additionally, IL-6, IL-8, INF-γ, IL-12p70 and TNFα were able to further characterise VHR sub-groups in terms of co-morbidities and primary or recurrent cardiovascular events. These results highlight the importance of evaluating immune markers in CVD risk assessment and demonstrate that a multimarker approach could make risk stratification in VHR individuals more effective and will help identify participants at higher MACE risk.

5.1 Introduction

Inflammation plays a major role in the initiation and complications of atherosclerosis which results in coronary artery disease, one of the major subtypes of cardiovascular disease (CVD). Targeting the immune system to treat CVD initiated with the Cardiovascular Inflammation Reduction Trial (CIRT), where a low dose of methotrexate was used, and the ongoing Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) where canakinumab is used to target interleukin 1 beta (IL-1β) (239). IL-1β is normally produced after activation by caspase 1 through the NLRP3 inflammasome and in the CANTOS trial, now in phase 3, canakinumab was shown to reduce recurrent events (in terms of heart attack, stroke and cardiovascular death) in participants who survived a heart attack. The lesson learned from these trails is that additional pro or anti-inflammatory mediators need to be explored in order to assess their value in predicting recurrent major adverse cardiovascular events (MACE) to potentially use them as a treatment target or as therapy in CVD.

Several inflammatory mediators are known to contribute to atherosclerosis, however, their role in primary and secondary CVD prevention needs further understanding. Some of those inflammatory mediators include interleukin 6 (IL-6) and interleukin 2 (IL-2) which were associated with the development of CVD (240,241). IL-6 is known to contribute to myocardial damage and chronic heart failure (242) whereas IL-2 causes severe depression of cardiac function. When IL-2 was combined to an interleukin 2 antibody complex it reduced cardiac remodelling following a myocardial infarction (243) and heart failure progression (244). On the

other hand, Interleukin 8 (IL-8) is known to maintain a proinflammatory environment around the culprit lesion leading to cardiac events (245) and levels were shown to be higher in unstable coronary artery disease (246). Moreover, Interferon γ (IFN-γ) is a major macrophage activating factor that induces the release to TNFα (247) and has consequently a major role in CVD (248). In contrast, Interleukin 10 (IL-10) has been associated with an anti-inflammatory response and a decreased risk in developing heart failure in patients post myocardial infarction (MI) after primary angioplasty. Nevertheless, IL-10 levels were positively associated with cardiovascular risk in the elderly without prior CVD events (249). It was shown that Interleukin 13 (IL-13) deficiency aggravated healing and remodelling in male mice after myocardial infarction (250). On the other hand, interleukin 4 (IL-4) was proposed as a potential therapeutical target for cardiac fibrosis (251) whereas interleukin 12 (IL-12) is known to have a controversial role in CAD (252,253). In addition, it is already well-established that TNFα levels are higher in patients with an acute myocardial infarction (AMI) (153,154) with higher levels in AMI patients compared to stable angina patients (165). These inflammatory markers have been investigated in separate studies that linked them to different clinical or biological aspects of CVD. Therefore, the role of such inflammatory markers in establishing an individual's cardiovascular risk is not clear. As biomarker research is moving towards a multimarker approach, several validated inflammatory protein panels are already commercialised. However, these protein panels need to be tested in clinical practice to assess whether they add knowledge to the current risk prediction tools. Therefore, this study evaluates the value of the MSD® proinflammatory panel which combines

10 proteins involved in the immune and inflammatory response in order to determine its utility in predicting cardiovascular risk in different participant groups.

5.2 Participants and Methods

Refer to **section 2.1** in **Chapter 2** for a detailed description of the participant recruitment process, the risk score used and the methods for blood processing.

5.2.1 Demographic information

Refer to **section 3.2.1** in **Chapter 3** for a detailed description of the demographic information that was collected in this study.

5.2.2 Measurement of inflammatory plasma protein levels

Refer to section **2.10 in Chapter 2.**

5.2.3 Measurement of *IL1B* gene expression

Refer to section **2.11, 2.12 and 2.13 in Chapter 2.**

5.2.4 Statistical methods

Statistical analysis was carried out as described in **section 3.2.7** in **Chapter 3**

5.3 Results

5.3.1 Populaition Demographics

Refer to section **3.3.1 in Chapter 3** and **Table 1 and 2 in Chapter 3**.

<u>Table 1:</u> INF-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, Il-4, IL-6, IL-8 and TNFα levels in participants at various levels of cardiovascular risk classified according to the SCORE risk chart.

	VHR	**MR**	**LR**	***p-value (non-adjusted)***	***p value (adjusted)****
Number of participants	*229*	*32*	*81*		
IFN-γ (pg/ml)	9.93 ± 42.02	8.93 ± 11.27	7.99 ± 11.68	0.199	0.024
IL-10 (pg/ml)	0.375 ± 0.600	0.256 ± 0.114	0.399 ± 1.17	0.428	0.139
IL-12p70 (pg/ml)	0.299 ± 0.345	0.208 ± 0.139	0.226 ± 0.162	0.022	0.199
IL-13 (pg/ml)	0.782 ± 0.611	0.828 ± 0.791	0.530 ± 0.368	0.002	0.018
IL-1β (pg/ml)	0.407 ± 1.052	0.194 ± 0.106	0.166 ± 0.117	0.091	0.087
IL-2 (pg/ml)	0.266 ± 0.275	0.211 ± 0.111	0.243 ± 0.321	0.102	0.245
IL-4 (pg/ml)	0.0323 ± 0.1451	0.0171 ± 0.0059	0.0186 ± 0.0145	0.566	0.459
IL-6 (pg/ml)	2.06 ± 2.80	0.930 ± 0.638	0.563 ± 0.424	<0.0001	0.001**
IL-8 (pg/ml)	8.16 ± 10.00	4.80 ± 2.72	3.01 ± 1.35	<0.0001	<0.0001
TNFα (pg/ml)	4.711 ± 2.453	3.056 ± 0.767	2.658 ± 0.729	p<0.0001	0.003
	VHR		**Non-VHR**	***p-value (non-adjusted)***	***p value (adjusted)****
Number of participants	*229*		*115*		
IFN-γ (pg/ml)	9.93 ± 42.02		8.22 ± 11.43	0.092	0.003
IL-10 (pg/ml)	0.375 ± 0.600		0.358 ± 0.990	0.189	0.39
IL-12p70 (pg/ml)	0.299 ± 0.345		0.220 ± 0.155	0.005	0.084
IL-13 (pg/ml)	0.782 ± 0.611		0.625 ± 0.542	0.003	0.05
IL-1β (pg/ml)	0.407 ± 1.052		0.185 ± 0.120	0.05	0.111
IL-2 (pg/ml)	0.266 ± 0.275		0.231 ± 0.272	0.028	0.775
IL-4 (pg/ml)	0.0323 ± 0.1451		0.018 ± 0.013	0.295	0.395
IL-6 (pg/ml)	2.06 ± 2.80		0.066 ± 0.512	<0.0001	<0.0001
IL-8 (pg/ml)	8.16 ± 10.00		3.50 ± 1.99	<0.0001	<0.0001
TNFα (pg/ml)	4.711 ± 2.453		2.770 ± 0.752	p<0.0001	<0.0001
IL-1β gene expression (2(-DcT)	0.0654 ± 0.0413		0.0335 ± 0.0272	0.003	

ANCOVA analysis was used to compare the cohorts. Two p values were calculated to assess the impact of the covariates on the analysis. All proteins were measured in the plasma except IL1B which represent mRNA levels. (LR: low risk; MR: moderate risk; IL-1β: Interleukin 1 beta; IL-2: Interleukin 2; IL-4: Interleukin4; IL-6: Interleukin 6; IL-8: Interleukin 8; IL-10: Interleukin 10; IL-12p70: Interleukin 12 subunit 70; IL-13: Interleukin 13; IFN-γ: Interferon gamma; non-VHR: non-very high risk; TNFα: Tumour necrosis factor alpha; VHR: very high risk. Proteins not in bold and in italic had very low limit of detection level and their values were not included in subsequent analysis.

**adjusted for age and gender and BMI for IL-6. For statistical analysis, all protein levels were transformed using a log transformation as values did not follow the normal distribution.*
*** MR vs. LR (p=0.028)*

5.3.2 INF-γ, IL-6 and IL-8 plasma protein levels were higher in VHR participants

In order to evaluate whether a panel combining 10 inflammatory proteins could add value to the existing CVD, risk assessment scores, a combination of proteins from the MSD® Proinflammatory Panel 1 were measured in individuals at various levels of CVD risk.

Results demonstrated that INF-γ (9.93 ± 42.02 pg/ml vs. 8.22 ± 11.43 pg/ml; $p<0.01$), IL-6 (2.06 ± 2.80 pg/ml vs. 0.066 ± 0.512 pg/ml; $p<0.0001$) and IL-8 (8.16 ± 10.00 pg/ml vs. 3.50 ± 1.99 pg/ml; $p<0.0001$) plasma protein levels were significantly higher in the VHR compared to the non-VHR group. Additionally, INF-γ ($p<0.05$), IL-6 ($p<0.001$) and IL-8 ($p<0.0001$) plasma levels significantly increased with increasing cardiovascular risk (Table 1 and Figure 1). Furthermore, IL-6 plasma levels were significantly higher in MR participants compared to LR participants (0.930 ± 0.638 pg/ml vs. 0.563 ± 0.424 pg/ml; $p<0.05$) (Table 1 and Figure 1b).

Regarding IL-13 and IL-4 measurements, IL-13 and IL-4 plasma levels were higher in VHR compared to non-VHR participants. Nevertheless, due to problems with the assay's low limit of detection as well as the assay's high coefficient of variation in some of the tested samples, IL-13 and IL-4 plasma levels could not be clinically interpreted. Most of IL-1β plasma levels were also below the limit of detection of the assay. However, in a small subset of participants that were randomly selected, *IL1B*

gene expression was analysed and levels were higher in VHR compared to non-VHR participants (0.0654 ± 0.0413 vs. 0.0335 ± 0.0272; p<0.01) (Table 1 and Figure 1d).

On the other hand, IL-10, IL-12p70, and IL-2 plasma levels did not show any significant difference in the studied cohorts.

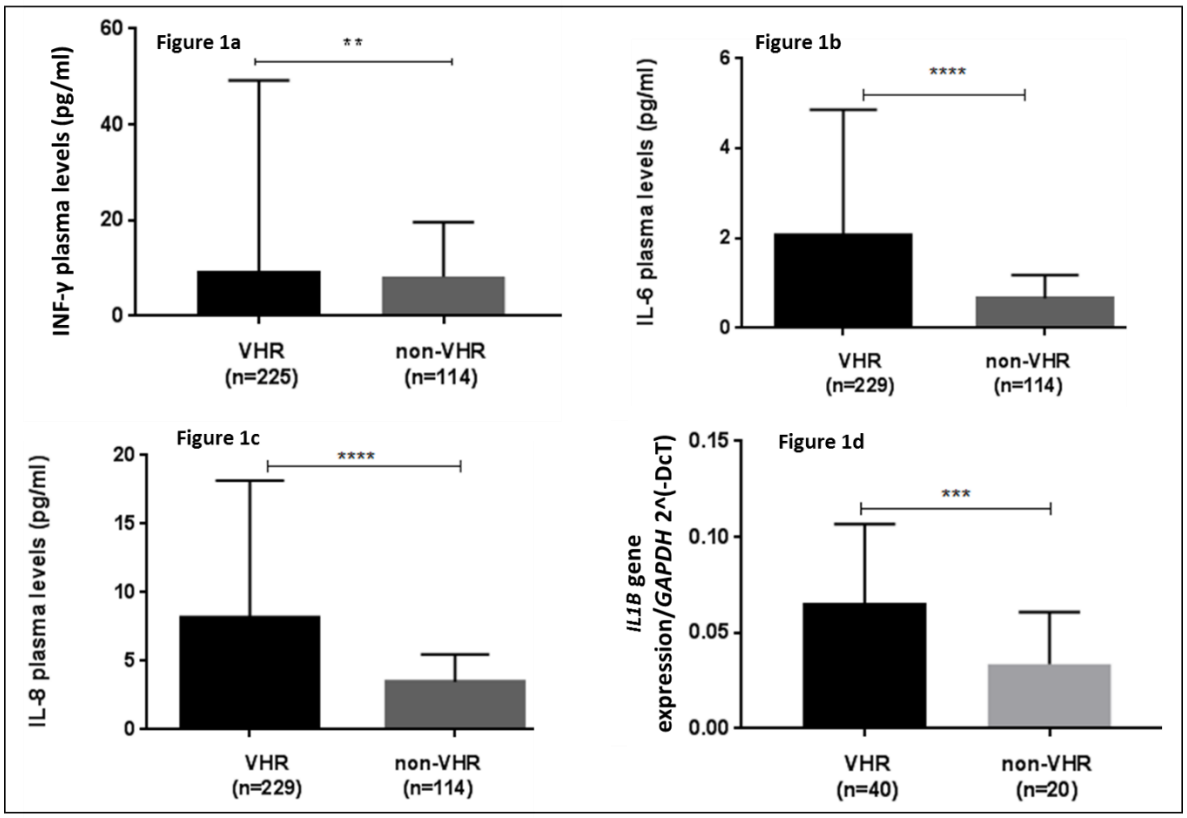


Figure 1: ***INF-γ, IL-6, IL-8 and IL-1β levels are higher in participants at very high cardiovascular risk.*** *INF-γ, IL-6 and IL-8 plasma protein levels were measured using the MSD® MULTI-SPOT Assay System.* IL-1β *mRNA levels were measured by quotative real-time PCR and normalised to GAPDH. Figure 1a: IFN-γ plasma levels are higher in VHR vs. non-VHR participants. Figure 1b: IL-6 plasma levels are higher in VHR vs. non-VHR participants. Figure 1c: IL-8 plasma levels are higher in VHR vs. non-VHR participants. Figure 1d: IL-1β gene expression levels are higher in VHR vs. non-VHR levels. IL-1β: Interleukin 1 beta; IL-2: Interleukin 2; IL-4: Interleukin4; IL-6: Interleukin 6; IL-8: Interleukin 8; IL-10: Interleukin 10; IL-12p70: Interleukin 12 subunit 70; IL-13: Interleukin 13; IFN-γ: Interferon gamma; non-VHR: non-very high risk; VHR: very high risk. Statistical analysis was performed by ANCOVA. ** p<0.01, ***p<0.001, ****p<0.0001*

5.3.3 INF-γ, IL-6, IL-8, IL12-p70 and IL-10 plasma protein levels differentiated between VHR participant subgroups

Proteins from the MSD ® Proinflammatory panel 1 were explored to assess whether VHR individuals could be further classified into subgroups according to their cardiovascular history and associated comorbidities.

5.3.3.1 Markers associated with acute cardiovascular events

Results demonstrated that IFN-γ and IL-6 plasma protein levels were higher in VHR participants with an acute coronary syndrome (ACS-VHR) compared to VHR elective percutaneous intervention participants (ELEC-VHR) (10.19 ± 53.03 pg/ml vs. 8.52 ± 9.12 pg/ml; $p<0.01$ and 2.75 ± 3.57 pg/ml vs. 1.20 ± 0.70 pg/ml; $p<0.001$ respectively). In addition, when evaluating the proteins associated with recurrent major adverse cardiovascular events (MACE), results showed that IFN-γ (20.88 ± 89.61 pg/ml vs. 4.52 ± 3.78 pg/ml; $p<0.01$), IL-12p70 (0.35 ± 0.44 pg/ml vs. 0.28 ± 0.42 pg/ml; $p<0.05$) and IL-8 (9.87 ± 10.92 pg/ml vs. 7.48 ± 12.31 pg/ml; $p<0.01$) plasma levels were higher in ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first cardiovascular event. Interestingly however, INF-γ plasma levels were lower in ACS-VHR participants admitted for their first cardiovascular event who had no previous cardiac events compared to ELEC-VHR participants with no previous cardiac events (4.59 ± 3.91 pg/ml vs. 7.79 ± 6.62 pg/ml; $p<0.0001$). In contrast, IL-6 plasma levels were higher in ACS-VHR participants admitted for their first cardiovascular event who had no previous cardiac events compared to ELEC-VHR participants with no previous cardiac events (2.75 ± 3.72 pg/ml vs. 1.16 ± 0.55 pg/ml; $p<0.01$).

5.3.3.2 Markers associated with medical and clinical management

Regarding VHR participants with a revascularisation strategy, data showed that IL-8 plasma levels were higher in VHR participants with a previous percutaneous coronary intervention (PCI) compared to VHR participants with no previous PCI (9.10 ± 8.82 vs. 7.72 ± 10.51 pg/ml; $p<0.01$). This trend was also observed regardless of whether VHR individuals were ACS-VHR or ELEC-VHR participants. In fact, IL-8 plasma levels were higher in ACS-VHR participants with a previous PCI compared to ACS-VHR participants with no previous PCI (8.802 ± 7.144 pg/ml vs. 8.914 ± 12.897 pg/ml; $p=0.80$) and in ELEC-VHR participants with a previous PCI compared to ELEC-VHR participants with no previous PCI (9.285 ± 9.798 pg/ml vs. 6.893 ± 3.651 pg/ml; $p=0.092$). Additionally, IL-8 plasma levels were also higher in VHR participants with a previous coronary artery bypass graft (CABG) compared to VHR participants with no previous CABG (10.40 ± 12.84 vs. 7.92 ± 9.66 pg/ml; $p<0.05$).

VHR participants with a previous PCI or CABG were more likely to be on statin (91.8%; $p<0.0001$, 100%; $p<0.0001$), anti-platelet (91.8%; $p<0.0001$, 86.4%; $p<0.0001$) and anti-hypertensive therapy (93.2%; $p<0.0001$, 90.9%; $p<0.0001$) and had lower levels of total cholesterol (3.73 ± 1.00 mmol/L vs. 4.40 ± 1.40 mmol/L; $p<0.001$ and 3.55 ± 0.90 mmol/L vs. 4.27 ± 1.34 mmol/L; $p<0.05$ respectively).

In addition, the present results demonstrated that IFN-γ (5.12 ± 5.31 pg/ml vs. 10.82 ± 45.61 pg/ml; $p<0.05$), IL-12p70 (0.27 ± 0.49 pg/ml vs. 0.31 ± 0.29 pg/ml; $p<0.01$) and IL-8 (8.03 ± 14.75 pg/ml vs. 8.22 ± 8.07 pg/ml; $p<0.05$) plasma levels were lower in VHR drug naïve participants compared to VHR participants on medication.

5.3.3.3 Markers associated with follow-up events and CVD comorbidities

The current data suggested that IL-6 plasma levels were associated with follow up MACE within 6 months ($p<0.05$).

Additionally, when it comes to VHR participants with CVD comorbidities, our results demonstrated that IL-6 plasma levels were higher in VHR participants with diabetes compared to VHR participants with no diabetes (2.32 ± 2.63 pg/ml vs. 2.00 ± 2.84 pg/ml; $p<0.05$). Interestingly, IL-6 plasma levels were higher in VHR participants who currently smoked ($p<0.05$). Moreover, IL-6 (3.30 ± 3.43 pg/ml vs. 1.83 ± 2.62 pg/ml; $p<0.0001$) plasma levels were higher in VHR participants with heart failure compared to VHR participants with no heart failure, whereas INF-γ (4.85 ± 3.34 pg/ml vs. 10.29 ± 43.23 pg/ml; $p<0.05$) plasma levels were lower.

Furthermore, IFN-γ (12.25 ± 52.66 pg/ml vs. 5.81 ± 5.35 pg/ml; $p<0.05$) and IL-12p70 (0.35 ± 0.44 pg/ml vs 0.24 ± 0.15 pg/ml; $p<0.05$) plasma levels were higher in VHR participants with hypertension compared to those with no hypertension. On the other hand, IL-8 plasma levels were higher in VHR participants with arthritis compared to those with no arthritis (13.01 ± 19.44 pg/ml vs. 7.28 ± 6.82 pg/ml; $p<0.01$) whereas IL-10 plasma levels were lower in VHR participants with dyslipidaemia (0.30 ± 0.32 pg/ml vs. 0.48 ± 0.85 pg/ml; $p<0.05$). (Table 2, Figure 2 and 3).

5.3.3.4 Markers associated with angiographic outcome

Protein from the MSD® proinflammatory panel were explored to assess whether they could determine the plaque burden in VHR participants. After carrying out a

multinomial logistic regression analysis (section 3.2.7 in Chapter 3), results showed that IL-12p70 plasma levels appeared to predict angiographic outcome with a p value nearly reaching significance (p=0.052). IL-12p70 plasma levels were the highest in participants with very severe coronary artery disease compared to participants who had an occlusion and those who had a triple vessel disease (0.3604 ± 0.5060 pg/ml, 0.3482 ± 0.4486 pg/ml and 0.2561 ± 0.1322 pg/ml respectively with p=0.052). Looking at this more closely, data showed that participants with the highest levels of IL-12p70 were more likely not to have had a previous MI and not to be on medication. Among VHR participants with a very severe coronary artery disease, 14.5 % had a previous MI and 33.3% were not on medication as opposed to VHR participants with occlusion among whom 24.4% had a previous MI and 14.6% were not on medication and VHR participants with triple vessel disease among whom 17.8% had a previous MI and 21.4% were not on medication.

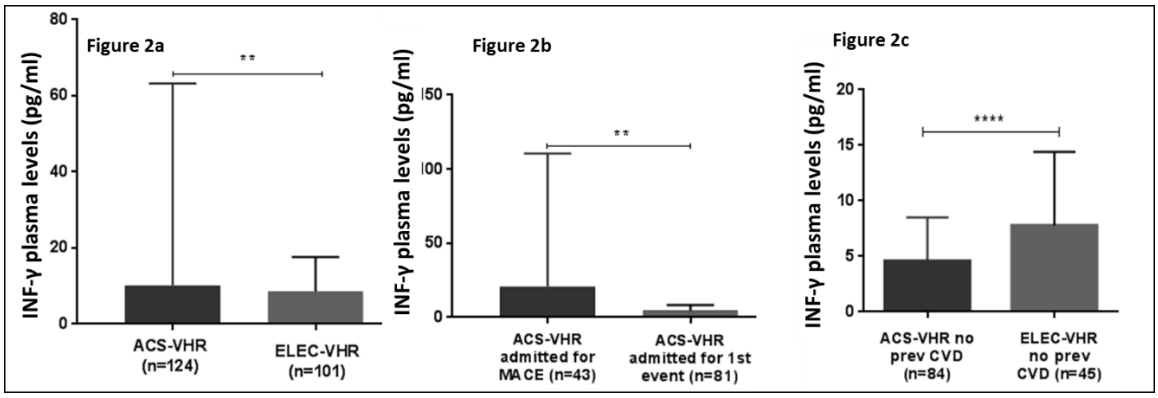


Figure 2: ***IFN-γ plasma protein levels in VHR participants****. INF-γ plasma protein levels were measured using the MSD® MULTI-SPOT Assay System. Figure 2a: INF-γ plasma levels were higher in ACS-VHR vs ELEC-VHR participants; Figure 2b: INF-γ plasma levels were higher in ACS participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first event; Figure 2c: INF-γ plasma levels were lower in ACS-VHR participants compared to ELEC-VHR participants with no previous CVD history. ACS: acute coronary syndrome participants; CVD: cardiovascular disease; ELEC: elective participants; CABG: coronary artery bypass graft; IFN-γ Interferon gamma; MACE: major adverse cardiovascular events, MI: myocardial infarction, PCI: percutaneous coronary*

*intervention; VHR: very high risk. Statistical analysis was performed by Student t-test for figure 2a and 2c and by ANCOVA for figure 2b. **p<0.01, ****p<0.0001*

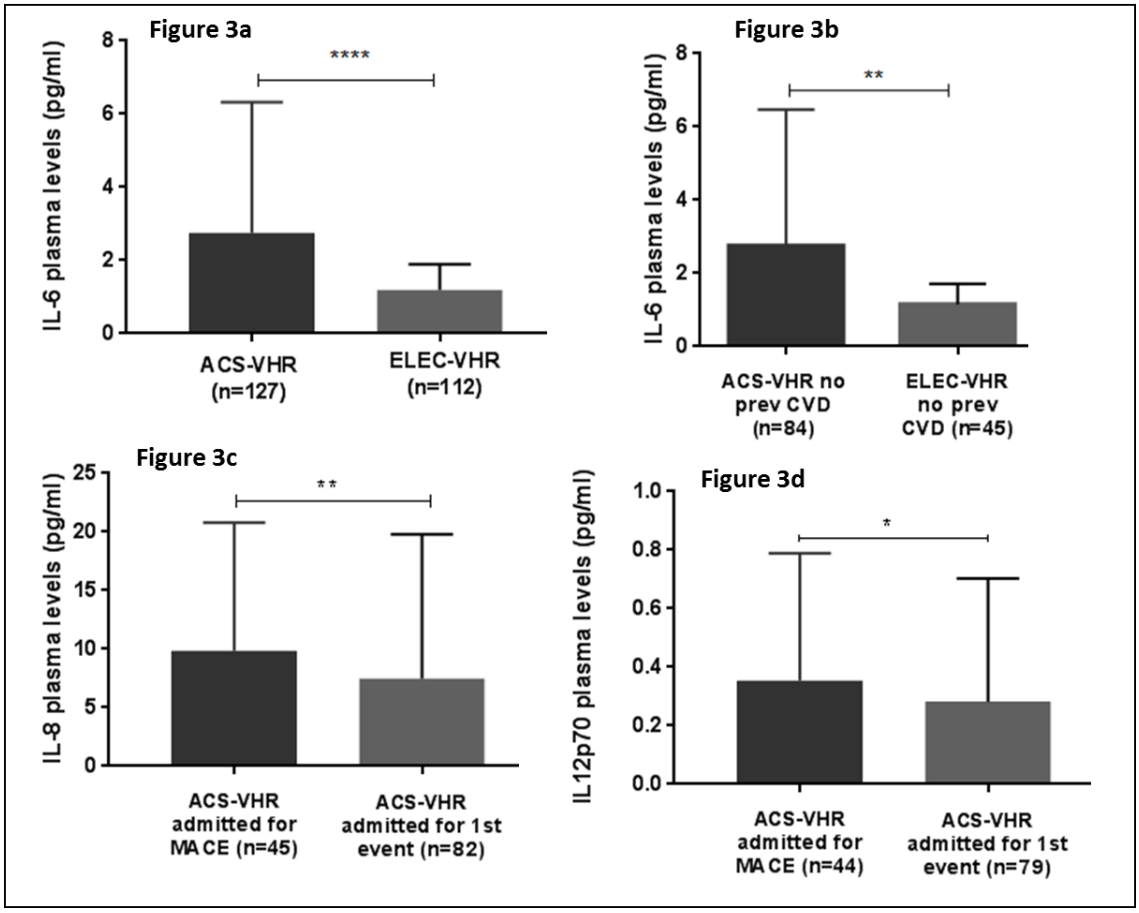


***Figure 3:* IL-6, IL-12p70 and IL-8 plasma protein levels in VHR participants**. *IL-6, IL-12p70 and IL-8 plasma protein levels were measured using the MSD® MULTI-SPOT Assay System. Figure 3a: IL-6 plasma levels were higher in ACS-VHR vs. ELEC-VHR participants; Figure 3b: IL-6 plasma levels were higher in ACS-VHR vs ELEL-VHR with no previous CVD history; Figure 3c: IL-8 plasma levels were higher in ACS-VHR participants admitted for MACE vs. ACS-VHR participants admitted for first CVD; Figure 3d: IL-12p70 plasma levels were higher in ACS-VHR participants admitted for MACE vs. ACS-VHR participants admitted for first CVD; ACS: acute coronary syndrome participants; ELEC: elective participants; CABG: coronary artery bypass graft; IFN-γ Interferon gamma; MACE: major adverse cardiovascular events, MI: myocardial infarction, PCI: percutaneous coronary intervention; VHR: very high risk. Statistical analysis was performed by Student t-test for figure 3a and 3b and by ANCOVA for figure 3c and 3d. *p<0.05; **p<0.01; ****p<0.000*

5.3.4 Assessing the value of a multimarker model in predicting cardiovascular risk.

In order to evaluate whether the aforementioned significant proteins from the MSD® proinflammatory panel were able to predict VHR or non/VHR group membership, a logistic regression analysis was performed (section 3.2.7 in Chapter 3). This showed that a panel combining INF-γ, IL-6, IL-8 and TNFα plasma levels was able to predict 85.8% of the VHR/non-VHR group membership ($p<0.0001$) as opposed to 79.3%, 75.2% and 76.9% when using IL-6, IL-8 and TNFα alone respectively. The r square of the combined model was 0.667 which suggests that 66.7% of the variability is predicted by the combined model.

5.3.5 A cluster analysis to further stratify VHR cohort using INF-γ, IL-6, IL-8 and TNFα plasma protein levels

To explore whether VHR individuals could be further stratified according to the levels of their inflammatory markers, a two-way cluster analysis was carried out. Using IFN-γ, IL-6, IL-8 and TNFα plasma levels generated two clusters within the VHR cohort. The choice of those proteins was based on their ability to identify VHR participants and their aptitude in further differentiating between VHR sub-groups. Participants in cluster 1 ($n=140$) had lower plasma levels of IFN-γ (4.68 ± 3.45 pg/ml vs. 17.28 ± 64.03 pg/ml; $p<0.0001$), IL-6 (1.24 ± 0.97 vs. 3.35 ± 4.04 pg/ml; $p<0.0001$), IL-8 (5.42 ± 2.66 pg/ml vs. 12.81 ± 15.00 pg/ml; $p<0.0001$) and TNFα (3.63 ± 0.98 pg/ml vs. 6.53 ± 3.06 pg/ml; $p<0.0001$) compared to cluster 2 ($n=85$). Cluster 2 also grouped older participants (68 ± 10 years vs. 64 ± 11 years; $p<0.01$), participants with a lower total cholesterol levels (3.93 ± 1.15 mmol/L vs. 4.39 ± 1.38 mmol/L; $p<0.01$), a higher systolic blood pressure (126 ±

21 mmHg vs. 134 ± 25 mmHg; p<0.05), higher levels of IL-10 (0.498 ± 0.873 pg/ml vs. 0.296 ± 0.346 pg/ml; p<0.0001), higher levels of IL-12p70 (0.369 ± 0.370 pg/ml vs. 0.257 ± 0.326 pg/ml; p<0.000), higher levels of IL-2 (0.318 ± 0.396 pg/ml vs. 0.233 ± 0.151 pg/ml; p<0.05) and higher levels of CRP (22.77 ± 48.35 mg/L vs. 7.30 ± 18.57 mg/L; p<0.01) compared to cluster 1. In addition, cluster 2 also had a higher percentage of VHR participants admitted for recurrent MACE (54.3% vs. 24.3%; p<0.0001), a higher percentage of VHR participants with previous MI (43.5% vs 24.3%; p<0.01), a higher percentage of VHR participants with previous PCI (42.4% vs. 25.7%; p<0.0001), a higher percentage of VHR diabetic participants (29.4% vs 12.9%; p<0.01) and a higher percentage of VHR participants with heart failure (24.7% vs. 10%; p<0.01) compared to cluster 1.

5.3.6 CRP plasma levels did not further differentiate between VHR participant sub-groups

Refer to **section 3.3.6** in **Chapter 3.**

5.3.7 Correlations between biomarker protein levels

To explore whether protein levels measured as part of the MSD® proinflammatory panel correlated with different variables explored in the study, a correlation analysis was performed (section 3.2.7 in Chapter 3). For a full correlation matrix, refer to **Table 6** in **section III** of the Appendix. Results indicated that IFN-γ plasma levels correlated positively with IL-10, IL-12p70, IL-2, IL-8 and TNFα plasma levels (r=0.284; p<0.0001, r=0.166; p<0.01, r=0.176; p<0.01, r=0.120; p<0.05 and r=282; p<0.0001 respectively). IL-6 plasma levels correlated positively with age, body mass index (BMI), IL-10, IL-8 and TNFα plasma levels (r=0.456; p<0.0001, r=225; p<0.0001, r=0.173; p<0.01, r=0.264;

p<0.001, and r=0.423; p<0.0001 respectively) and negatively with total cholesterol levels (r=-0.200; p<0.0001). IL-8 plasma levels correlated positively with age, systolic blood pressure, IFN-γ, IL-10, IL-12p70, IL-2, IL-6 and TNFα plasma levels (r=0.368; p<0.0001, r=0.122; p<0.05, r=0.120; p<0.05, r=0.122; p<0.05, r=0.199; p<0.0001, r=0.162; p<0.01, r=0.264; p<0.0001 AND R=0.486; P<0.0001 respectively) and negatively with total cholesterol levels (R=-0.234; p<0.0001). IL-12p70 plasma levels correlated positively with age, INF-γ, IL-10, IL-2, IL-8 and TNFα plasma levels (r=0.151; p<0.01, r=0.166; p<0.01, 0.143; p<0.01, r=0.221; p<0.0001, r=0.199; p<0.0001 and r=0.300; p<0.0001 respectively). TNFα plasma levels correlated positively with C reactive protein (CRP) plasma levels, age and BMI (p<0.01, p<0.0001 and p<0.05 respectively) and negatively with cholesterol levels (p<0.0001). Refer to Table 4 in **section 3.3.7** of **Chapter 3**.

Table 2: ***Inflammatory protein levels in very high risk participants***

	IFN-γ (pg/ml)		*p value*	IL-10 (pg/ml)		*p value*	IL-12p70 (pg/ml)		*p value*	IL-13 (pg/ml)		*p value*	IL-1β (pg/ml)		*p value*	IL-2 (pg/ml)		*p value*	IL-4 (pg/ml)		*p value*	IL-6 (pg/ml)		*p value*	IL-8 (pg/ml)		*p value*
Demographic and History *(n; %)*	**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
Gender																											
Male *(180; 78.6)*	10.15	44.89	0.71	0.39	0.66	0.59	0.28	0.28	0.43	0.75	0.57	0.57	0.46	1.21	0.35	0.27	0.29	0.20	0.04	0.16	0.06	2.20	3.02	0.30	8.04	8.44	0.71
Female *(49; 21.4)*	6.90	5.43		0.33	0.32		0.37	0.52		0.89	0.73		0.26	0.18		0.24	0.19		0.01	0.01		1.56	1.72		8.61	14.46	
Diabetes mellitus																											
Present *(44; 19.2)*	7.06	6.87	0.96	0.39	0.45	0.40	0.23	0.12	0.21	0.83	0.51	0.33	0.26	0.16	0.85	0.28	0.20	0.60	0.02	0.02	0.40	2.32	2.63	0.04	9.25	15.42	0.70
Absent *(185; 80.8)*	10.01	44.11		0.37	0.63		0.32	0.38		0.77	0.63		0.44	1.17		0.26	0.29		0.04	0.17		2.00	2.84		7.90	8.25	
Heart failure																											
Present *(36; 15.7)*	4.85	3.34	0.03	0.35	0.43	0.90	0.26	0.14	0.97	0.70	0.60	0.23	0.28	0.15	0.96	0.24	0.14	0.80	0.01	0.01	0.48	3.30	3.43	<0.0001	8.03	6.55	0.53
Absent *(193; 84.3)*	10.29	43.23		0.38	0.63		0.31	0.37		0.80	0.61		0.44	1.17		0.27	0.29		0.04	0.16		1.83	2.62		8.18	10.54	
Renal function																											
GFR < 60 *(45; 20)*	7.09	5.95	0.50	0.42	0.47	0.59	0.26	0.18	0.76	0.77	0.88	0.26	0.28	0.17	0.88	0.31	0.21	0.26	0.02	0.01	0.12	2.38	2.79	0.50	7.54	6.47	0.83
GFR > 60 *(180; 80)*	10.14	44.75		0.37	0.64		0.31	0.38		0.78	0.54		0.45	1.19		0.26	0.29		0.03	0.16		2.01	2.83		8.35	10.81	
Hypertension																											
Present *(128; 58.9)*	12.25	52.66	0.04	0.40	0.69	0.98	0.35	0.44	0.02	0.83	0.65	0.23	0.47	1.34	0.61	0.28	0.34	0.93	0.04	0.19	0.68	1.96	2.41	0.73	8.65	11.29	0.20
Absent *(101; 44.1)*	5.81	5.35		0.34	0.46		0.24	0.15		0.72	0.55		0.31	0.23		0.24	0.16		0.02	0.01		2.19	3.24		7.53	8.09	

Dyslipidaemia																											
Present *(134; 58.5)*	7.20	8.19	0.56	0.30	0.32	0.01	0.28	0.34	0.28	0.79	0.63	0.82	0.34	0.32	0.29	0.25	0.15	0.70	0.04	0.19	0.67	1.83	2.28	0.30	7.50	4.77	0.22
Absent *(95; 41.5)*	12.63	61.15		0.48	0.85		0.32	0.35		0.78	0.59		0.48	1.53		0.29	0.38		0.02	0.01		2.39	3.40		9.09	14.45	
Arthritis																											
Present *(35; 15.3)*	24.82	99.70	0.24	0.49	1.11	0.97	0.41	0.53	0.13	1.05	0.91	0.04	0.92	2.18	0.04	0.35	0.58	0.25	0.09	0.33	0.55	2.85	3.62	0.09	13.01	19.44	0.01
Absent *(194; 84.7)*	6.61	5.94		0.35	0.45		0.28	0.30		0.73	0.52		0.26	0.20		0.25	0.17		0.02	0.02		1.92	2.61		7.28	6.82	
Depression																											
Present *(43; 18.8)*	6.92	5.56	0.17	0.41	0.68	0.68	0.28	0.24	0.64	0.83	0.61	0.71	0.26	0.30	0.38	0.21	0.11	0.45	0.01	0.01	0.25	1.86	1.85	0.33	9.74	15.39	0.21
Absent *(186; 81.2)*	10.04	44.15		0.37	0.58		0.30	0.37		0.77	0.61		0.44	1.15		0.28	0.30		0.04	0.16		2.11	2.98		7.79	8.29	
Present or previous cancer																											
Present *(27; 11.8)*	8.55	7.75	0.31	0.38	0.47	0.84	0.27	0.17	0.92	0.65	0.45	0.25	0.25	0.18	0.75	0.24	0.15	0.21	0.02	0.01	0.21	2.76	2.81	0.06	7.86	7.01	0.73
Absent *(202; 88.2)*	9.57	42.32		0.37	0.62		0.30	0.36		0.80	0.63		0.43	1.11		0.27	0.29		0.03	0.16		1.97	2.79		8.20	10.35	
Smoking status																											
None *(66; 30.3)*	7.25	9.72	0.53	0.31	0.25	0.52	0.29	0.23	0.61	0.79	0.71	0.56	0.30	0.33	0.54	0.25	0.15	0.72	0.08	0.29	0.34	1.99	2.51	0.02	7.86	8.20	0.47
Ex-smoker *(110; 50.4)*	12.50	56.53		0.43	0.74		0.31	0.44		0.73	0.48		0.56	1.57		0.29	0.37		0.02	0.02		2.00	3.03		8.95	12.35	
Current *(42; 19.3)*	5.37	4.35		0.38	0.65		0.27	0.23		0.92	0.72		0.32	0.10		0.21	0.11		0.02	0.02		2.53	2.88		6.87	5.37	
Pharmacotherapy																											
Statin therapy																											
Present *(165; 72.7)*	10.82	46.85	0.08	0.38	0.62	0.98	0.32	0.39	0.09	0.83	0.63	0.08	0.46	1.18	0.14	0.28	0.31	0.07	0.04	0.17	0.76	1.82	2.27	0.03	8.93	11.18	0.003

Absent *(62; 27.3)*	5.94	5.94		0.36	0.55		0.25	0.18		0.66	0.55		0.21	0.15		0.22	0.16		0.02	0.01		2.77	3.84		6.21	5.68	
Antiplatelet therapy																											
Present *(154; 67.8)*	11.46	48.52	0.03	0.37	0.61	0.32	0.33	0.40	0.003	0.88	0.67	0.001	0.46	1.16	0.09	0.28	0.31	0.18	0.04	0.18	0.54	1.93	2.46	0.13	8.40	8.46	0.04
Absent *(73; 32.1)*	5.37	5.07		0.39	0.58		0.23	0.18		0.56	0.36		0.19	0.15		0.22	0.16		0.02	0.02		2.39	3.44		7.75	12.81	
Antihypertensive therapy																											
Present *(172; 76.8)*	10.59	45.75	0.14	0.38	0.61	0.51	0.33	0.39	0.002	0.84	0.62	0.003	0.44	1.14	0.66	0.28	0.30	0.06	0.04	0.16	0.69	1.93	2.46	0.23	8.86	10.97	0.002
Absent *(55; 24.2)*	5.95	6.07		0.37	0.58		0.20	0.11		0.56	0.51		0.25	0.16		0.22	0.17		0.01	0.01		2.52	3.69		6.07	5.90	
Antianginal therapy																											
Present *(88; 38.8)*	8.26	9.40	0.03	0.34	0.40	0.32	0.32	0.40	0.23	0.92	0.78	0.05	0.38	0.33	0.02	0.24	0.16	0.34	0.07	0.25	0.40	1.50	1.51	0.03	8.90	8.48	0.03
Absent *(139; 61.2)*	10.25	50.85		0.40	0.70		0.29	0.31		0.68	0.43		0.44	1.42		0.28	0.33		0.02	0.02		2.44	3.34		7.74	10.92	
Diuretic therapy																											
Present *(56; 24.7)*	18.36	79.25	0.12	0.47	0.94	0.72	0.34	0.40	0.11	0.78	0.48	0.86	0.63	1.93	0.49	0.31	0.48	0.30	0.02	0.01	0.73	2.52	2.90	0.09	8.61	8.78	0.12
Absent *(171; 75.3)*	6.55	7.52		0.35	0.44		0.29	0.32		0.79	0.65		0.32	0.29		0.25	0.16		0.04	0.18		1.93	2.77		8.05	10.44	
Drug naïve																											
Present *(54; 23.7)*	5.12	5.31	0.02	0.33	0.58	0.67	0.27	0.49	0.01	0.55	0.28	0.01	0.24	0.19	0.38	0.22	0.14	0.43	0.01	0.01	0.49	2.44	3.66	0.24	8.03	14.75	0.04
Absent *(174; 76.3)*	10.82	45.61		0.39	0.61		0.31	0.29		0.85	0.66		0.45	1.17		0.28	0.30		0.04	0.16		1.96	2.49		8.22	8.07	
Clinical Variables																											
ACS-VHR *(127; 55.5)*	10.19	53.03	0.002	0.38	0.65	0.38	0.31	0.43	0.24	0.76	0.65	0.31	0.48	1.41	0.64	0.27	0.33	0.89	0.02	0.02	0.72	2.75	3.57		8.33	11.85	0.24

ELEC-VHR *(102; 44.5)*	8.52	9.12		0.37	0.54		0.29	0.20		0.81	0.57		0.32	0.35		0.26	0.17		0.05	0.23		1.20	0.70	<0.0001	7.95	7.12	
Previous MI																											
Present *(73; 31.9)*	7.98	6.47	0.09	0.35	0.31	0.91	0.29	0.16	0.18	0.73	0.56	0.28	0.28	0.23	0.76	0.28	0.19	0.54	0.02	0.01	0.25	1.82	1.81	0.83	8.14	6.28	0.18
Absent *(156; 68.1)*	10.12	47.92		0.39	0.70		0.31	0.40		0.81	0.64		0.50	1.38		0.26	0.31		0.04	0.18		2.17	3.16		8.17	11.35	
Previous PCI																											
Present *(73; 31.9)*	8.47	9.66	0.10	0.33	0.25	0.53	0.29	0.20	0.36	0.84	0.70	0.47	0.34	0.36	0.94	0.26	0.18	0.59	0.06	0.26	0.32	1.73	2.01	0.34	9.10	8.82	0.02
Absent *(56; 68.1)*	9.90	47.83		0.40	0.70		0.30	0.39		0.75	0.55		0.46	1.35		0.27	0.31		0.02	0.02		2.22	3.10		7.72	10.51	
Previous CABG																											
Present *(22; 9.6)*	36.20	127.85	0.04	0.65	1.38	0.54	0.41	0.60	0.21	0.88	0.53	0.35	1.27	2.99	0.12	0.43	0.73	0.30	0.02	0.02	0.36	2.30	3.29	0.55	10.40	12.84	0.05
Absent *(207; 90.4)*	6.69	7.34		0.35	0.44		0.29	0.31		0.77	0.62		0.29	0.28		0.25	0.16		0.03	0.15		2.04	2.75		7.92	9.66	
Previous MI, PCI or CABG																											
Present *(100; 43.7)*	14.36	60.11	0.008	0.40	0.70	0.55	0.31	0.32	0.09	0.84	0.69	0.42	0.51	1.36	0.25	0.30	0.38	0.35	0.05	0.22	0.78	1.89	2.35	0.20	9.30	9.83	0.003
Absent *(129; 56.3)*	5.71	5.23		0.36	0.51		0.29	0.36		0.73	0.53		0.26	0.18		0.24	0.15		0.02	0.02		2.19	3.11		7.27	10.08	
Diagnosis upon admission																											
Stable Angina (85; 37.1)	8.82	9.37	0.02	0.39	0.57	0.79	0.33	0.41	0.53	0.85	0.72	0.09	0.27	0.35	0.59	0.25	0.16	0.75	0.06	0.24	0.58	1.20	0.71	<0.0001	9.12	12.67	0.09
Unstable Angina (21; 9.2)	6.39	6.89		0.48	0.66		0.25	0.19		0.51	0.27		0.28	0.23		0.24	0.12		0.01	0.01		1.16	0.77		6.70	3.77	
NSTEMI < 1 week (77; 33.6)	13.23	68.52		0.39	0.75		0.30	0.37		0.75	0.54		0.59	1.73		0.30	0.41		0.02	0.02		3.29	3.65		7.68	9.13	
STEMI < 1 week (7; 3.0)	5.58	4.52		0.25	0.08		0.29	0.17		0.43	0.26		0.35	0.12		0.19	0.08		0.01			6.73	7.36		13.07	14.17	

Other (39; 17.03)																												
ACS-VHR participants admitted for MACE																												
Present *(45; 35.4)*	20.88	89.61	0.01	0.51	0.99	0.18	0.35	0.44	0.05	0.89	0.87	0.26	0.70	2.01	0.75	0.35	0.51	0.06	0.02	0.01	0.45	3.07	3.68	0.51	9.87	10.92	0.01
Absent (82; 64.6)	4.52	3.78		0.31	0.32		0.28	0.42		0.68	0.47		0.28	0.17		0.23	0.14		0.02	0.02		2.58	3.52		7.48	12.31	
Participants with no previous MI, PCI or CABG																												
ACS-VHR *(84, 65.1)*	4.59	3.91	<0.0001	0.31	0.32	0.30	0.28	0.42	0.09	0.67	0.47	0.10	0.28	0.17	0.17	0.23	0.16	0.48	0.02	0.02	0.58	2.75	3.72	0.01	7.56	12.23	0.25
ELEC-VHR *(45, 34.9)*	7.79	6.62		0.46	0.75		0.31	0.23		0.85	0.63		0.22	0.20		0.25	0.15		0.02	0.01		1.16	0.55		6.74	3.62	
Prognostic variable																												
Follow-up MACE																												
Within 6 months *(5; 2.2)*	5.00	2.37	0.85	0.28	0.10	0.89	0.21	0.13	0.35	0.65	0.38	0.95	0.26	0.09	0.54	0.24	0.16	0.84	0.02	0.01	0.76	4.66	3.78	0.02	7.68	2.82	0.74
After 6 months *(11; 4.8)*	8.22	8.86		0.48	0.75		0.22	0.16		0.63	0.21		0.41	0.13		0.28	0.16		0.01	0.01		2.33	1.77		7.28	2.66	
Absent *(213; 93.0)*	9.61	41.23		0.37	0.60		0.31	0.35		0.79	0.63		0.41	1.09		0.27	0.28		0.03	0.15		1.99	2.80		8.22	10.35	

Highlighted cells refer to p values <0.05. (ACS: acute coronary syndrome participants; CABG: Coronary artery bypass surgery; ELEC: elective participants; GFR: Glomerular filtration rate; MACE: major adverse cardiovascular events MI: Myocardial Infarction; NSTEMI: non-ST elevation myocardial infarction; PCI: percutaneous coronary intervention; STEMI: ST elevation myocardial infarction; VHR: very high risk).

5.4 Discussion

The present investigation identified potential candidate biomarkers from the MSD® proinflammatory panel that could improve the current CVD risk assessment tools. Furthermore, these proteins demonstrated a superior value to CRP measurement in further stratifying individuals at VHR of MACE risk. However, some of the tested proteins had a low limit of detection and a high coefficient of variations when tested in plasma samples. Such panels, once optimised, can be measured in large prospective longitudinal studies to assess their additional value in assessing CVD risk.

5.4.1 IFN-γ, IL-6, IL-8 and TNFα identified individuals at cardiovascular risk

Levels of IN-γ, IL-6, IL-8 and TNFα have been explored in separate studies, however, their collective role in identifying individuals at various levels of cardiovascular risk hasn't been evaluated yet. In the present study IFN-γ, IL-8, IL-6, and TNFα plasma protein levels significantly differentiated between VHR and non-VHR participants. In addition, IL-6 plasma protein levels could also distinguish between MR and LR participants. Previous evidence has showed that IFN-γ plays an important role in atherosclerosis and plaque stability (254) however, IFN-γ plasma levels haven't been previously measured in participants at different levels of cardiovascular risk. Additionally, IL-8 is known to be involved in maintaining an inflammatory environment surrounding the plaque (245) and IL-8 levels have been shown to be higher in unstable coronary artery disease (246). Furthermore, IL-6 levels have been linked to cardiovascular risk for over a decade (255) and many studies have associated IL-6 with CVD risk (256) but also with melanoma (257) and influenza (258). Similarly, although TNFα plasma levels have been measured in individuals with CVD (153), TNFα is a systematic inflammatory cytokine and is also associated with several other inflammatory diseases (259) and infections (260). This

highlights one of the major problems associated with biomarker development which is non-specificity since levels tend to be associated with other diseases and therefore complicate the diagnostic. One of the solutions to this problem is combining markers in an inflammatory panel which will then compensate for the non-specificity that individual biomarkers might face (261,262). The important clinical value of such a multimarker panel is reflected in a logistic regression model which demonstrated that combining IFN-γ, IL-6, IL-8 and TNFα plasma level measurement resulted in an 85.8% accurate prediction of the VHR/non-VHR group membership as opposed to 79.3%, 75.2% and 76.9% when using IL-6, IL-8 and TNFα alone respectively. Furthermore, when age, gender and BMI were added to this model, the accuracy of predicting cohort membership increased to 91.7% ($p<0.0001$). This highlights the significant value of using a multimarker approach in predicting cardiovascular risk. In addition, some markers in the panel showed to be useful in further stratifying individuals at very high risk of CVD as discussed in section 5.4.2.

On the other hand, the value of IL-1β as a therapeutic target in CVD has proven to be effective as the ongoing Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial is showing promising results (263). Furthermore, IL-1β has been linked to atherothrombotic disease and plaque destabilisation as well as adverse remodelling after a myocardial infarction (MI) (264). Therefore, keeping in mind the importance of IL-1β as a therapeutic target in CVD, its additional value to a multimarker CVD panel was assessed. Nevertheless, in the present study, the measurement of plasma IL-1β resulted in many values below the limit of detection of the MSD® assay (refer to the Methods section II.X in the appendices). In fact, measuring IL-1β levels in the plasma has proven to be quite challenging as it required the use of ultra-sensitive

assays in previous studies (265). Therefore, to compensate for this analytical problem and to evaluate whether IL-1β has a potential value in CVD risk assessment, *IL1B* gene expression was measured. Results showed that *IL1B* mRNA levels were higher in VHR compared to non-VHR participants. Therefore, despite the clinical value demonstrated by IL-1β gene expression evaluation in CVD risk assessment, including IL-1β protein measurement in a panel test might require optimisation.

5.4.2 IFN-γ, IL-6, IL-8 and IL12-p70 can further differentiate between VHR participants

The present data indicated that IFN-γ and IL-6 plasma levels were higher in ACS-VHR compared to ELEC-VHR participants. These results are in line with previous reports of higher INF-γ and IL-6 levels in participants with acute MI (266,267). IL-6 is a pro-inflammatory cytokine involved in acute phase response and, in addition to its release from T cells and macrophages, has been shown to be produced by muscles (268). High IL-6 levels have been previously reported in participants with STEMI (269) (also shown in the present study - Table 2) which suggests that the source of IL-6 after an MI could also originate from the heart muscle. Interestingly, it has been reported that muscle produced IL-6 has anti-inflammatory effects and dampers TNFα response (270). This could mean that the release of IL-6 following an acute phase response partially aims to counteract a critical increase in inflammation. Moreover, IL-6 plasma levels were associated with MACE within a 6-months follow up period which has been previously reported (266,267,271). However, our study shows that IL-6 plasma levels were particularly higher in participants who developed MACE within 6 months after admission which is a novel finding.

In terms of recurrent cardiovascular events, the present study showed that IL-12p70 and IL-8 plasma levels were higher in ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first cardiovascular event and in VHR participants who were on medication compared to participants who were not. Participants on medication were more likely to have had a previous MI, PCI or CABG. This shows first that IL-12p70 and IL-8 could be possible markers for acute events in participants with a CVD history and second, that medical treatment fails to decrease the levels of IL-12p70 and IL-8 plasma levels. Interestingly, INF-γ plasma levels were lower in ACS-VHR participants admitted for their primary cardiovascular event compared to ELEC-VHR participants but were higher in ACS-VHR participants admitted for their recurrent MACE. This effect could be due to medical treatment since participants admitted for recurrent MACE were on medication and INF-γ plasma levels were higher in VHR individuals on medication.

Is a matter of fact, IL-12p70 is a heterodimer of IL-12 formed by alpha helices coded by two separate IL-12 genes (IL-12 p35 and IL-12 p40). IL-12 levels have been shown to be related to adverse outcomes in participants with STEMI (272) and to endothelial dysfunction (273) and in the present study, IL-12p70 levels were higher in ACS-VHR participants admitted for recurrent MACE. However, IL-12/23 inhibitors, majorly used in psoriasis and psoriasis arthritis treatment, have been shown to cause MACE (274) which highlights a need to further understand the consequences of completely inhibiting IL-12 in CVD. Furthermore, in this present study, IL-12p70 levels seemed to predict angiographic outcome and were found to be higher in participants with a very severe plaque burden who were also more likely to be drug naïve and to have no previous CVD history. These findings are in line with a previous report where IL-12 levels were higher

in participants with three affected coronary arteries compared to those with one affected coronary artery (272).

Based on INF-γ, IL-6, IL-8 and TNFα plasma levels of a cluster analysis could further stratify the VHR participants into 2 clusters. Cluster 2 grouped a higher percentage of VHR individuals with a severe CVD history in terms of recurrent MACE, previous MI or PCI, diabetes and heart failure. Cluster 2 also grouped older participants with a high body mass index (BMI) who have high inflammatory markers with high plasma levels of C reactive protein (CRP), IL-10, IL-12p70 and IL-2. This shows that participants belonging to cluster 2 require a closer follow up as they are at higher risk of MACE due to higher levels of inflammatory proteins and a severe cardiac history. Therefore, the present cluster analysis demonstrates that, in order to accurately predict the risk of recurrent MACE, a multimarker panel needs to account for the extent of increases in biomarker levels and needs to combine risk factors that assess an individual's cardiac history and comorbidities.

5.4.3 Strengths and Limitations

Refer to **section 3.4.3** in **Chapter 3**

In terms of potential biases resulting from the biochemical measurements used in the present study, it is important to consider that IL-13, IL-1β and IL-4 plasma levels were below the limit of detection of the used assay. However, IL-1β gene expression levels were measured in a subset of participants. There were 5 values that were below the limit of detection for INF-γ assay, 5 for the IL-10 assay, 36 for the IL-2 assay, 1 for the IL-6 assay, 1 for the IL-8 assay and 12 for the IL-12p70 assay which also had a high coefficient of variation. Refer to the Methods section II.X in the appendices.

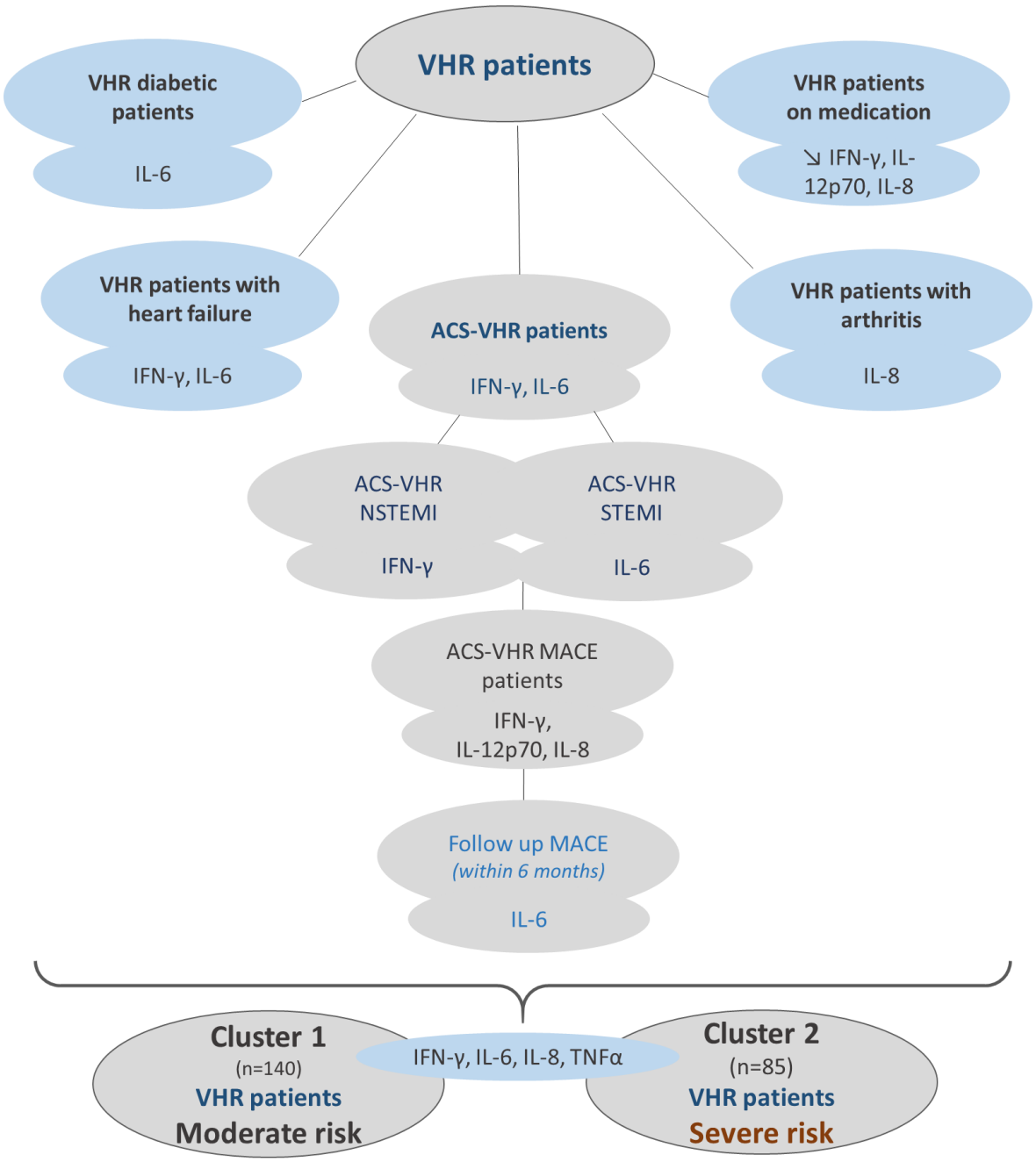


Figure 4: ***Proposed summary diagram highlighting the role of INF-γ, IL-6, IL-8 and IL-12p70 in cardiovascular risk stratification.*** *ACS-VHR: acute coronary syndrome participants; MACE: major adverse cardiovascular events, NSTEMI: non-ST segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction; VHR: very high risk*

5.5 Conclusion

This study shows that IL-6, IL-8, INF-γ and IL-1β can differentiate between individuals at various levels of CVD risk. Additionally, IL-6, IL-8, INF-γ and IL-12p70 can further stratify the VHR group. A panel combining such inflammatory markers could make risk stratification in these VHR participants much more effective and may identify individuals at higher risk of developing MACE. The results of this study need to be replicated in larger cohorts in prospective longitudinal studies in order to define the steps needed for the introduction of such panels in clinical practice.

Chapter 6

Biomarker Discovery set:

A proteomic approach for identifying individuals at various levels of cardiovascular risk

Abstract

Background: Biomarker discovery in cardiovascular disease (CVD) is moving towards a multimarker rather than a single marker approach. Since multiple inflammatory and immune pathways are involved in CVD, measuring one marker could be seen as too simplistic and is unlikely to capture an individual's susceptibility to develop first or recurrent major adverse cardiovascular events (MACE). Current traditional risk scoring systems are not ideally predictive as many patients calculated to have high scores do not suffer from CVD while others found to have low scores present with cardiovascular events. In this current study, using a traditional scoring system to assign scores to individuals at risk of CVD, we aimed to investigate whether a panel combining a set of 184 inflammatory proteins, known to be involved in immunity, atherosclerosis, plaque rupture and thrombosis could add value to this risk prediction tool and help further stratify individuals with CVD.

Methods: Participants were recruited from the cardiac catheterisation laboratory or by email advertisement. Group 1 was defined as very high risk participants (VHR) with a 10-year risk SCORE ≥10% risk of fatal CVD. Group 2 were defined as low, moderate and high risk participants (non-VHR) with a 10-year risk SCORE <10% risk of fatal CVD. Proteins from the cardiovascular panel II and III were measured in the plasma using multiplex proximity extension assays (PEA) by Olink Proteomics®.

Results: A total of 297 participants were recruited consecutively. The VHR group (n=208) consisted of acute coronary syndrome VHR (ACS-VHR) (n=113) and elective percutaneous intervention participants (ELEC-VHR) (n=95). The non-VHR group (n=89) consisted of low risk (LR) (n=61) and moderate risk (n=28) participants. A total of 72 proteins including MMP7 ($p<0.0001$), MMP12 ($p<0.0001$), REN ($p<0.001$), GDF-15

(p<0.01), PON3 (p<0.01) and CNTN1 (p<0.0001) were able to differentiate between VHR and non-VHR participants. A total of 77 proteins including ADAMTS-13 (p<0.0001), VEGF-D (p<0.0001), BOC (p<0.0001), PDL-2 (p<0.0001), protein AMBP (p<0.05), OPN (p<0.0001) and TRAP (p<0.0001) were able to identify ACS-VHR participants admitted for their first cardiovascular event. A total of 40 proteins including TNFSF13B (p<0.01), IL18-BP (p<0.01), TNFRSF11A (p<0.01), BOC (p<0.01), TNFR2 (p<0.01) and EGFR (p<0.05) identified ACS-VHR participants admitted for recurrent MACE. A total of 79 proteins including KIM1 (p<0.0001), IL-1RT1 (p<0.0001), Gal-3 (p<0.0001), REN (p<0.0001), VSIG2 (p<0.0001), FABP4 (p<0.001), GIF (p<0.001), PON3 (p<0.01) and HO-1 (p<0.05) characterised VHR participants with diabetes whereas a total of 71 proteins including TRAIL-R2 (p<0.0001), FGF-21 (p<0.001), ADM (p<0.0001), TNFRS11A (p<0.0001), CTRC (p<0.001) and SRC (p<0.05) characterised VHR participants with heart failure.

Conclusion: This proteomic approach evaluating 184 proteins from two CVD protein panels highlights a number of novel proteins that were able to differentiate between individuals at various levels of CVD risk. In addition, a number of distinct proteins further stratified VHR participants in relation to their CVD history (whether they had a primary or a recurrent MACE) or according to their co-morbidities (diabetes, heart failure, chronic kidney disease). Such protein panels need to be further investigated and tested in larger cohorts in order to fully evaluate their utility in stratifying individuals at risk MACE and providing them with unique proteomic signature to characterise their disease.

6.1 Introduction

Biomarker discovery in cardiovascular disease (CVD) faces several increasing challenges. Co-morbidities are prevalent amongst those with CVD, including conditions such as diabetes mellitus, heart failure, chronic kidney disease, metabolic disorders and also depression (275–278). Since these pathologies share common biological pathways, measuring one specific biomarker may be seen as too simplistic.

The search for CVD biomarkers began in the mid-1960s when creatine kinase and its cardiospecific isoform creatine kinase-MB were established as indicators of acute myocardial damage (279). Later, cardiac troponins emerged as a biomarker based on which participants admitted with an acute coronary syndrome (ACS) were classified (280,281) and natriuric peptides (NP) as biomarkers for heart failure (282). Despite years of research, very few CVD biomarkers have managed to make it into clinical practice, mainly due to issues in relation to assay specificity, standardisation and legal considerations (283).

The Framingham Study, which started in 1950, (284), initiated the development of several risk scoring systems that incorporated several biomarkers such as such as systolic blood pressure and cholesterol levels in order to assess CVD risk in an individual with no prior CVD history (285). Framingham risk scores (90), as well as others, such as the Systemic Coronary Risk Evaluation (SCORE) (83) and the Prospective Cardiovascular Münster (PROCAM) study (286) risk scores are accepted risk prediction tools to guide clinical decision-making. While these scores can inform treatment decisions, they leave ample room for improvement as they have been extensively criticised (95). This is because current risk scores use risk factors to establish CVD risk, however, traditional risk factors do not explain most of CVD risk. In fact, most events occur in participants

with an average risk score who are mistakenly considered to be at intermediate or low risk because they have none or only one of the CVD risk factors (287). In contrast, many patients who are classified as high-risk individuals do not experience a cardiovascular event even in the long term (288). This suggests that improved risk prediction models are necessary.

The complexity of CVD (289) demonstrates that one single biomarker is unlikely to capture the individual predisposition to the disease. However, improving CVD risk scores using new biomarkers has proven a major challenge. For example, C-reactive protein improves CVD risk prediction in the general population, but its predictive value is limited because of its strong correlation with other risk factors, such as body mass index and smoking (290) and it is also well-known that C-reactive protein is elevated in several inflammatory conditions apart from CVD (291). Due to CVD multifactorial causes, biomarker research is quickly moving towards a multimarker approach. The interest in generating multimarker scores that use a composite of several biomarkers (measured in parallel) to predict disease risk and patient outcomes is increasing (292–296). Biomarkers belonging to different pathways involved in atherosclerosis could be measured collectively to provide a better understanding of the underlying and ongoing inflammation and the moment it reaches a critical point when a vulnerable plaque is more likely to rupture and an ACS is more likely to occur. This approach would allow the identification of individuals at very high risk and help treat them more efficiently with the available resources in a context where CVD is now concomitant with obesity, hypertension, diabetes mellitus and an aging population.

In the present study, using the European risk SCORE chart, we investigated whether a set of 184 inflammatory proteins, known to be involved in immunity, atherosclerosis,

plaque rupture and thrombosis, could add value to this risk prediction model and help further stratify participants with CVD.

6.2 Participants and Methods

Refer to **section 2.1** in **Chapter 2** for a detailed description of the participant recruitment process, the risk score used and the methods for blood processing.

6.2.1 Demographic information

Refer to **section 3.2.1 in Chapter 3** for a detailed description of the demographic information that was collected in this study.

6.2.2 Method for analysing plasma samples using the Proximity Extension Assay (PEA) technology - Proseek® Multiplex provided by O-Link Proteomics (CVDII and CVDIII)

Refer to section **2.14 in Chapter 2 and Figure 12 and 13 in section 2.14 in Chapter 2.**

6.2.3 Statistical methods

Statistical analysis was carried out as described in **section 3.2.7** in **Chapter 3**

In addition, body mass index (BMI) was adjusted for when it significantly contributed to the variability in the measured proteins. Protein levels that were significantly different between the compared cohorts were selected. A logistic regression was used to examine whether the totality of the significant proteins could effectively predict the cohort membership. An ideal candidate biomarker would have measurements that yield different bell curves for each group of participants with different means and minimal overlap and would enable the assignment of a new measurement to one of the two

groups with confidence. Therefore, volcano plots were created for each two cohorts that were compared between each other. For each protein, minus Log(10) of the p value was plotted against the Log(2) of the difference in average NPX of the measured protein between the compared cohorts. Using the proteins that were statistically significant among our cohorts, we performed a principal component analysis (PCA) to examine how many components the measured proteins formed and whether these components were inter-correlated in terms of biological pathways. Using proteins which levels were statistically significant when comparing VHR participants with MACE and VHR participants with no MACE, VHR participants were further stratified. A two-way cluster analysis was performed to explore how many clusters the VHR participants form. Differences in those clusters were then explored using a crosstab chi square as well as an ANOVA. A multinomial logistic regression analysis was then performed to investigate whether the selected proteins can effectively predict the severity of the plaque burden. Pearson's correlation was used to evaluate the correlation between variables. Statistical significance was defined as values of $p<0.05$ in general. However, when conducting an ANCOVA statistical significance was set at <0.07.

6.3 Results and Discussion

In order to assess the value of proteomic research in CVD risk assessment, two CVD protein panels (CVDII and CVDIII) combining a total of 184 proteins were measured by multiplex proximity extension assay (PEA) technology. The measured proteins are part of pathophysiological pathways implicated in global inflammation, immune response and cell adhesion, among many others (Figure 12 and 13 in section 2.14 of Chapter 2), and most of them are widely expressed in the body. This proteomic approach identified several novel individual biomarkers and biomarker clusters that differentiated between

participants at various levels of CVD risk. Several promising biomarkers were also able to further stratify participants with an established CVD at risk of major adverse cardiovascular events (MACE).

6.3.1 Biomarkers of cardiovascular risk

6.3.1.1 Participant recruitment

A total of 297 participants were recruited consecutively over a period of two years. The very high risk (VHR) (n=208) participants consisted of acute coronary syndrome (ACS-VHR) (n=113) and elective percutaneous intervention (ELEC-VHR) participants (n=95). The non-VHR (n=89) participants consisted of low risk (LR) (n=61) and moderate risk (n=28) participants. Table 1 and 2 provide the full descriptive statistics of the population. The mean age of the VHR participants at study entry was 65 years and 20% were women. A proportion of 56% had a history of hypertension, 19% were diabetic and 19% were current smokers. The mean age of the non-VHR participants was 46 years and 70% were women. A proportion of 13% had hypertension (MR=28) and 3% were current smokers.

<u>Table 1:</u> Population demographics of participants at various levels of cardiovascular risk classified according to the SCORE risk chart.

	VHR	**MR**	**LR**	***p-value (One way ANOVA)***	**Non-VHR**	***p-value (t-test)***
Number of participants	208	28	61		89	
Age (years)	65	57	41	<0.0001	46	<0.0001
Male (n; %)	167; 80.3	15; 53.6	12; 19.7	<0.0001 *(Chi 2)*	27; 30.3	<0.0001 *(Chi 2)*
Female (n; %)	41; 19.7	13; 46.4	49; 80.3		62; 69.7	
Weight (Kg)	86.5 ± 19.5	83.1 ± 18.9	74.5 ± 14.9	<0.0001	77.3 ± 16.7	<0.0001
Height (cm)	171.2 ± 9.4	168.6 ± 10.5	166.7 ± 8.2	0.004	167.3 ± 9.0	0.001
BMI (Kg/m^2)	29.2 ± 6.5	29.3 ± 6.6	26.9 ± 5.8	0.045	27.7 ± 6.1	0.059
Systolic BP (mmHg)	132 ± 24	140 ± 23	118 ± 14	<0.0001	124 ± 21	0.016

Diastolic BP (mmHg)	73 ± 12	82 ± 17	74 ± 12	0.004	77 ± 14	0.018
Total Cholesterol (mmol/L)	4.2 ± 1.3	5.0 ± 1.3	5.01 ± 0.90	<0.0001	5.0 ± 1.0	<0.0001
LDL Cholesterol (mmol/L)	2.3 ± 1.0	2.9 ± 1.1	2.8 ± 0.9	<0.0001	2.8 ± 0.9	<0.0001
HDL Cholesterol (mmol/L)	1.2 ± 0.5	1.6 ± 0.4	1.6 ± 0.4	<0.0001	1.6 ± 0.4	<0.0001
TG (mmol/L)	1.8 ± 2.2	1.2 ± 0.5	1.1 ± 0.7	0.019	1.1 ± 0.7	0.005
CRP (mg/L)	12.6 ± 33.7	3.0 ± 2.5	2.4 ± 3.6	0.026	2.5 ± 3.5	0.007

One way ANOVA was used to compare the VHR, MR and LR cohorts. Student t-test was used to compare VHR to non-VHR. (BMI: body mass index; BP: blood pressure; CRP: C-reactive protein; HDL: high density lipoprotein; LDL: low density lipoprotein; LR: low risk; MR: moderate risk VHR: very high risk; non-VHR: non-very high risk; TG: triglycerides).

***Table 2:* Medical history and lifestyle characteristics of participants at various levels cardiovascular risk classified according to the SCORE risk chart.**

	VHR	**MR**	**LR**	*p-value (Chi 2)*	**Non-VHR**	*p-value (Chi 2)*
Number of participants	208	28	61		89	
	n; %				***n; %***	
Hypertension	117; 56.3	8; 28.6	4; 6.6	<0.0001	12; 13.5	<0.0001
Diabetes	39; 18.8	0; 0	0; 0		0; 0	
Dyslipidaemia	119; 57.2	19; 67.9	27; 44.3	0.078	46; 51.7	0.226
GFR<60	44; 21.1	1; 4.0	2; 3.4	0.001	3; 3.6	<0.0001
Arthritis	34; 16.3	5; 17.9	1; 1.6	0.01	6; 6.7	0.026
Depression	34; 19.2	4; 14.3	7; 11.5	0.337	11; 12.4	0.15
Employment status	57; 32.6	19; 76.0	60; 98.4	<0.0001	80; 91.9	<0.0001
Current smokers	38; 19.3	2; 7.4	1; 1.6	0.002	3; 3.4	<0.0001
Ex-smokers	99; 50.2	8; 29.6	26; 42.6	<0.0001	35; 34	<0.0001
Exercise	89; 62.2	21; 80.8	47; 78.3	0.029	69; 77.5	0.008
Alcohol consumption	101; 59.8	16; 61.5	52; 86.7	0.001	70; 79.1	0.002

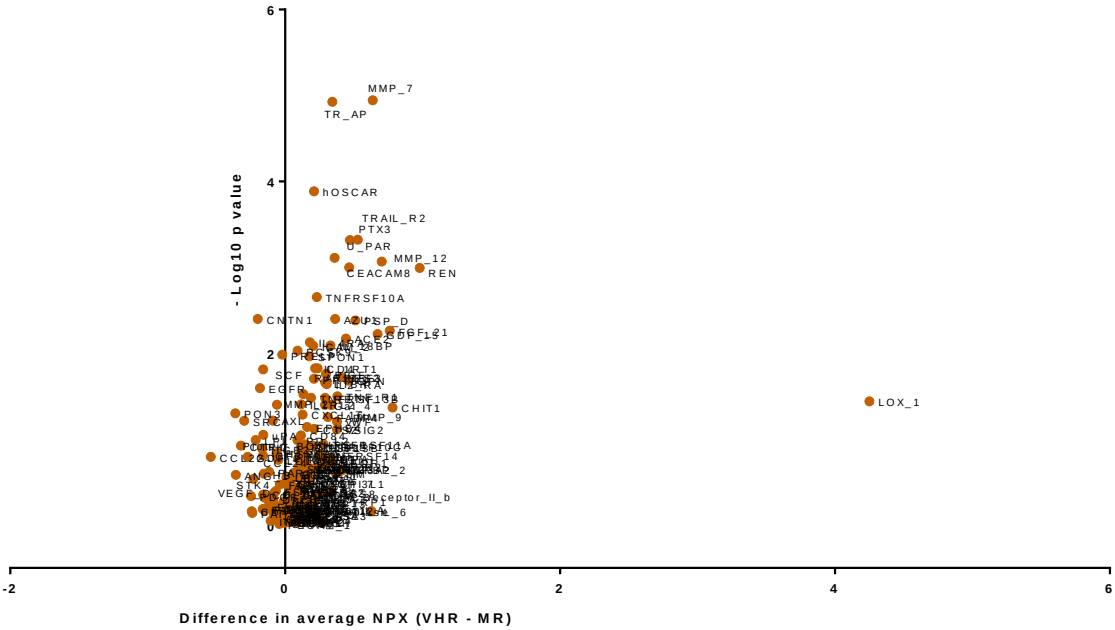
Pearson's chi square test was used to compare VHR, MR and LR groups and the VHR and non-VHR groups. (VHR: very high risk; MR: moderate risk; LR: low risk; non-VHR: non-very high risk GFR: Glomerular filtration rate).

6.3.1.2 Selection of potential proteins that differentiated between different cardiovascular risk groups

In order to select the proteins that significantly differentiated between two cohorts, mean NPX values were calculated for each protein in each cohort (VHR, MR, LR and non-VHR cohorts) and p values were generated when comparing two cohorts. Figure 1, 2, 3 and 4 are volcano plots representing the -Log10 of the p value plotted against the difference in the average NPX for each protein in each cohort (VHR/MR, VHR/LR, MR/LR and VHR/non-VHR respectively). The proteins that are at the top left or right side of the plot are the ones that show a strong statistical difference as well as a high difference between the protein means in the compared cohorts. It is noteworthy to mention that each 1 NPX value correspond to a 2-fold increase in the protein concentration. **Table 1** in **section III** of the appendix shows the list of proteins that were statistically different when compared across risk groups, the p values as well as the NPX mean and difference. Below is the list of significant proteins that were able to differentiate between two risk groups and that were explored in the context of the current literature.

Among 49 proteins, those that strongly differentiated between the VHR and MR cohorts were Matrix metalloproteinase-7 (MMP7) ($p<0.0001$), Tartrate-resistant acid phosphatase type 5 (TRAP) ($p<0.0001$), Lectin-like oxidized LDL receptor 1 (LOX-1) ($p<0.05$) and Osteoclast-associated immunoglobulin-like receptor (hOSCAR) ($p<0.0001$) (Figure 1). MMP7 in a metalloproteinase known to break the extracellular matrix and has been suggested to contribute to plaque instability in atherosclerosis (297). LOX-1 is known bind, internalise and degrade oxidised low-density lipoprotein and has been reported to be a marker for atherosclerosis related events (298). However, there is little

evidence in the literature of the role of TRAP and hOSCAR in CVD and their role should be further investigated in prospective studies. Interestingly, hOSCAR have been associated with osteoporosis (299), however TRAP is known to be expressed by macrophages and increased expression has been recently linked to cancer (300).



<u>Figure 1:</u> Volcano plot representing the p value against the difference in mean NPX between VHR and MR participants. *-Log10 of the p value was plotted against the difference between the average of the NPX value (VHR-MR). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). NPX: normalised protein ratio*

Some of the proteins that strongly differentiated between the VHR and the LR cohorts include MMP7 (p<0.0001), Lactoylglutathione lyase (GLO1) (p<0.0001), Lectin-like oxidized LDL receptor 1 (LOX-1) (p<0.0001), Carcinoembryonic antigen related cell adhesion molecule 8 (CEACAM8) (p<0.001), and Matrix metalloproteinase-12 (MMP12) (p<0.01) (Figure 2). Polymorphisms in *GLO1* have been associated with vascular diseases (301) whereas MMP12 has been suggested as a candidate molecule for the prevention and treatment of cardiometabolic diseases (302) and has also been linked to pulmonary

disease (303). However, there is little evidence in the literature on the role of CEACAM8, an adhesion molecule produced by granulocytes (304), in CVD and the mechanisms behind this interaction needs further understanding.

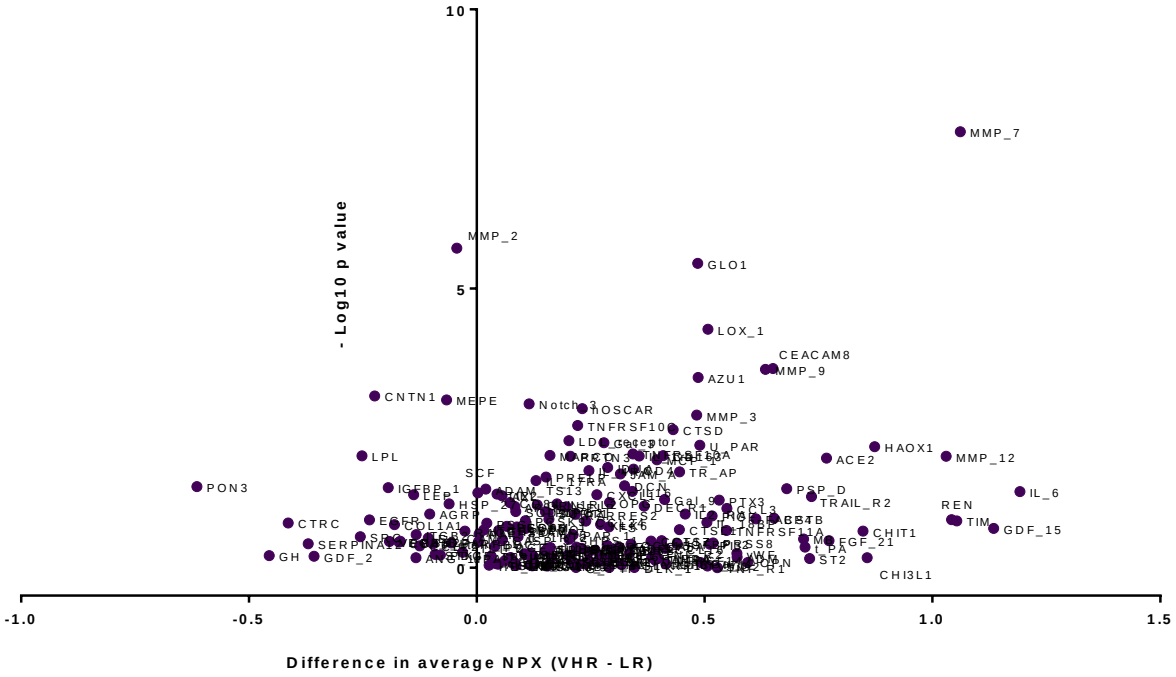


Figure 2: ***Volcano plot representing the p value against the difference in mean NPX between VHR and LR participants.*** *-Log10 of the p value was plotted against the difference between the average of the NPX value (VHR-LR). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). NPX: normalised protein ratio*

The proteins that strongly differentiated between the MR and the LR cohorts were LOX-1 (p<0.05), Pappalysin-1 (PAAPA) (p<0.05), Tumour necrosis factor receptor superfamily member 11A (TNFRSF11A) (p<0.05), cathepsin D (CTSD) (p<0.05) (Figure 3). PAPPA cleaves insulin like growth factor binding proteins and has been recently shown to be a potential marker of vulnerable plaques (295) whereas CTSD has previously been associated with MACE (305). However, TNFRSF11A and is known to regulate the interactions between T cells and dendritic cells (306) and have not been investigated before in participants with CVD.

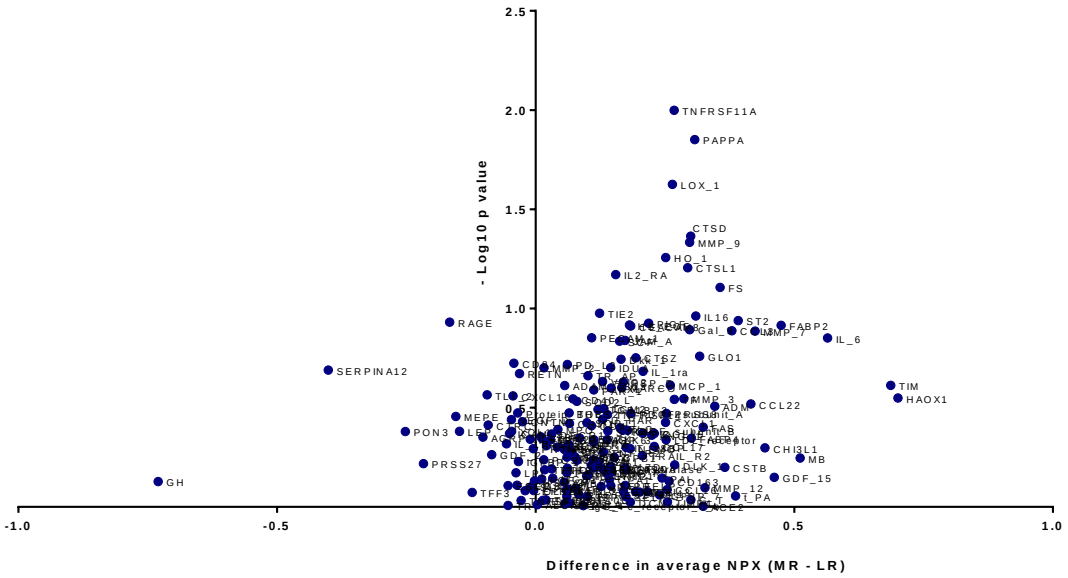
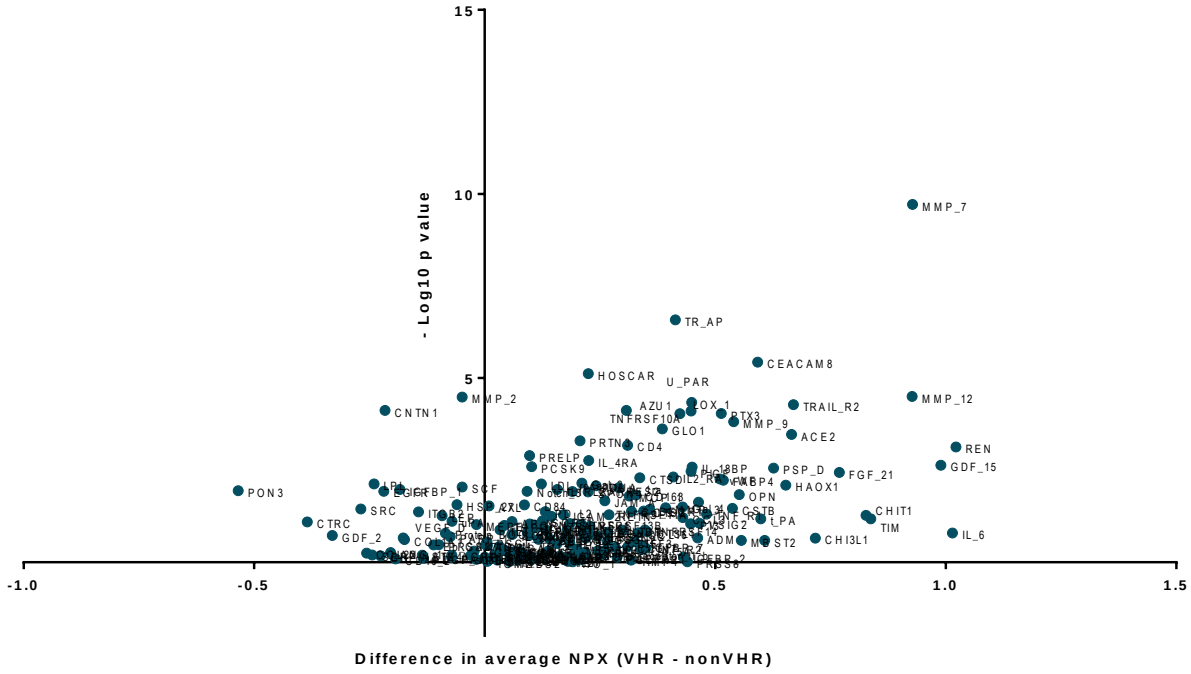


Figure 3: ***Volcano plot representing the p value against the difference in mean NPX between MR and LR participants.*** *-Log10 of the p value was plotted against the difference between the average of the NPX value (MR-LR). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). NPX: normalised protein ratio*

The best candidate proteins that differentiated between the VHR and the non-VHR cohorts were Matrix metalloproteinase-7 (MMP7) (p<0.0001), Matrix metalloproteinase-12 (MMP12) (p<0.0001), Renin (REN) (p<0.001), Growth/differentiation factor 15 (GDF-15) (P<0.01), Paraoxonase (PON3) (p<0.01) and Contactin-1 (CNTN1) (p<0.0001) (Figure 4). REN plasma activity has been recently linked to increased risk of MACE and congestive heart failure in participants with high systolic blood pressure (307). GDF-15 has been proposed as a new biomarker for CVD, stable coronary artery disease (CAD), ACS and heart failure (308). In addition, the role of the paraoxonase enzymes in CVD has been outlined especially through their involvement in the lipid metabolism (309) while the association between CNTN1 and new CVD onset has been also previously highlighted (122). Nevertheless, there are no studies that have evaluated PON3 plasma levels in participants with CVD.



<u>Figure 4:</u> Volcano plot representing the p value against the difference in mean NPX between VHR and non-VHR participants. *-Log10 of the p value was plotted against the difference between the average of the NPX value (VHR-nonVHR). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). NPX: normalised protein ratio*

Among the aforementioned proteins, it appears that MMP7 and LOX-1 were able to differentiate between more than two risk groups and their role in CVD has been previously described (297,298). However, in the present study, these proteins showed to have an important potential in CVD risk assessment and should be further explored in prospective studies as candidate biomarkers. In addition, CNTN1 and PON3 were both lower in VHR compared to MR ($p<0.01$; $p<0.05$), LR ($p<0.01$; $p<0.05$) and non-VHR participants ($p<0.0001$; $p<0.05$) suggesting that these two proteins might play a protective role in CVD which needs further understanding.

Among the other proteins that also showed to significantly differentiate between CVD risk groups (VHR/MR, VHR/LR, MR/LR and VHR/nonVHR) (Table 3), some haven't been previously measured in CVD individuals and to date, there is not enough literature

on the mechanisms behind their interaction with CVD. These proteins included Hydroxyacid Oxidase 1 (HAOX1), Azurocidin 1 (AZU1), Alpha-L-Iduronidase (IDUA), Macrophage receptor with collagenous structure (MARCO), Prolargin, (PRELP), Interleukin 17 Receptor A (IL17-RA), Neurogenic locus notch homolog protein 3 (Notch 3), CD84, Matrix extracellular phosphoglycoprotein (MEPE), Cathepsin Z cardiovascular (CTSZ), 2,4 Dienoyl-CoA reductase (DERC1), Galectin 4 (Gal4), Interleukin-18-binding protein (IL18BP), Integrin Subunit Beta 2 (TGB2), Programmed cell death 1 ligand 2 (PD-L2), Spondin 1 (SPON1), Proto-oncogene tyrosine-protein kinas (SRC), Lymphotoxin Beta Receptor (LTBR) and TNF Receptor Superfamily Member 13B (TNFRSF13B). As a matter of fact, despite little evidence in the literature associating these proteins with CVD, they are included in the cardiovascular disease panel II (CVDII) and III (CVDIII) provided by Olink Proteomics®. However, some of these proteins have been shown to be involved in inflammatory response, immune response, MAPK cascade, proteolysis, response to hypoxia, response to peptide hormone, wound healing, catabolic processes and chemotaxis (Figure 12 and 13 in section 2.14 in Chapter 2). Therefore, their inclusion in the panel is based on exploratory data that suggested a possible role in CVD.

In order to visualize the general inflammatory trend between VHR and non-VHR individuals, a heat map was generated (Figure 5). On the other hand, to investigate whether some proteins were able to differentiate between more than two risk groups, Venny 2.0 online tool was used. The results in Table 3 and Figure 6 represent the proteins that were unique or in common between risk groups (Venny 2.0, (Oliveros, J.C. (2007-2015) Venny - http://bioinfogp.cnb.csic.es/tools/venny/index.html). This demonstrated that LOX-1 and MMP9 were able to distinguish between all three risk groups. However, between these two proteins, LOX-1 had a higher sensitivity compared

to MMP9 as the p values and the difference in NPX mean levels generated by LOX-1 protein levels were higher when comparing two risk groups.

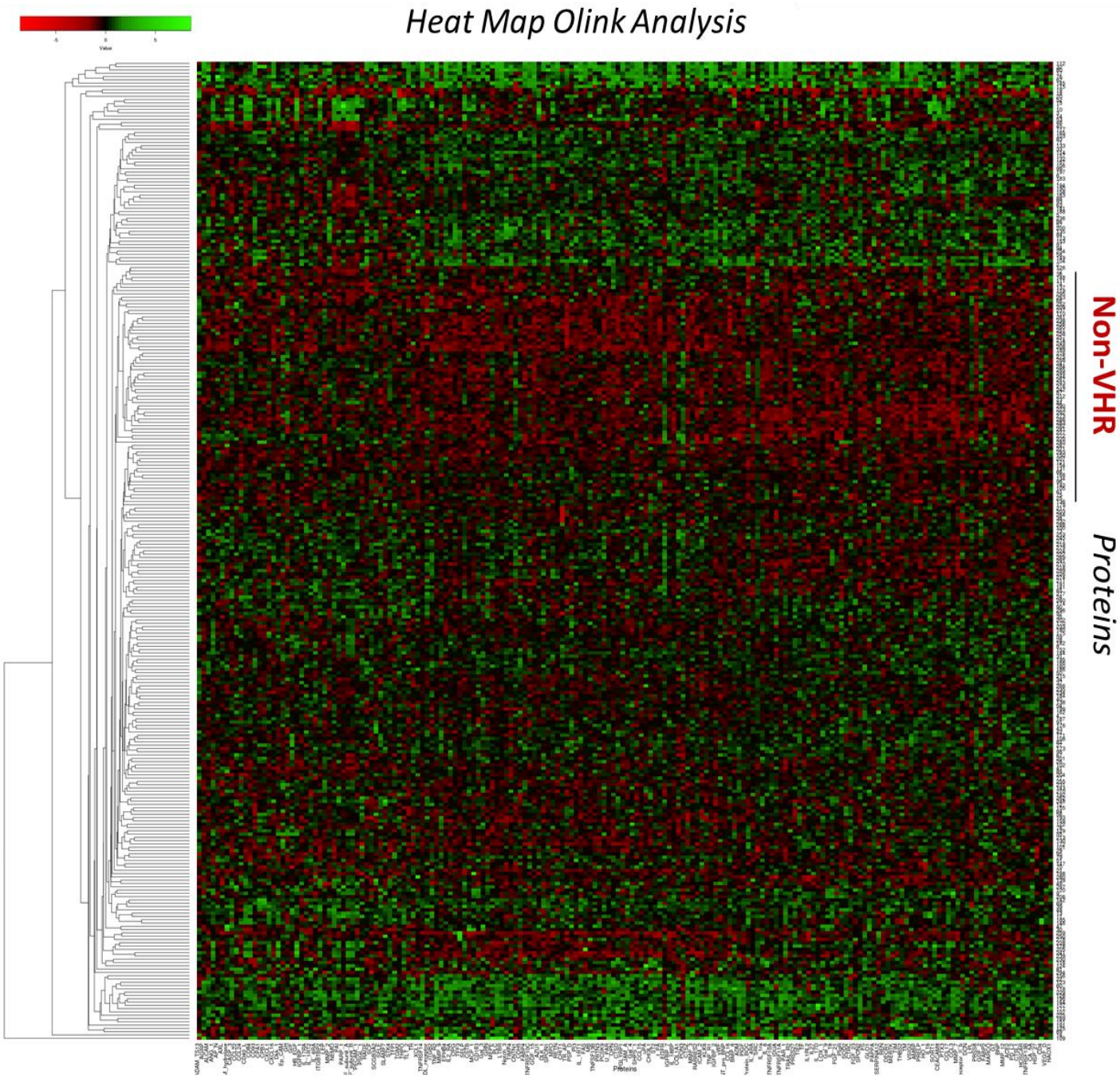


Figure 5: ***Heat map representing normalized protein levels (z-score) in the VHR and non-VHR cohort.*** *This heatmap was generated using the online tool www2.heatmapper.ca/expression. The green colour represents high protein expression and the red colour represents low protein expression. The clustering method applied was set to average linkage and the distance measurement method was set to Euclidian. The non-VHR participants tended to cluster together. The list of proteins are at the bottom of the heat map and the participants are on the right side of the heat map. Non-VHR: non very high risk participants.*

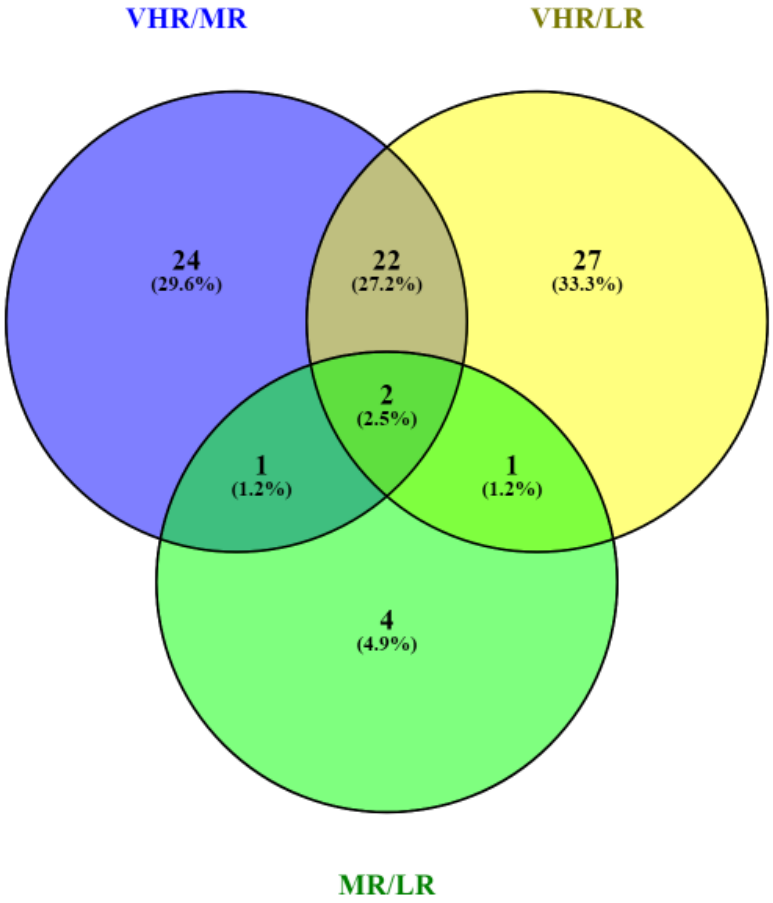


Figure 6: ***Diagram generated by Venny 2.0.*** *This diagram represents the number of proteins that differentiated between two risk groups and were unique to those two risk groups (24 proteins for VHR/MR, 27 proteins for VHR/LR and 4 proteins for MR/LR). It also represents the number of proteins that differentiated between two risk groups and were common between two risk groups (22 proteins that differentiated between VHR/MR and VHR/LR, 1 protein that differentiated between VHR/LR and MR/LR and 1 protein that differentiated between MR/LR and VHR/MR). Finally, it represents the 2 proteins that differentiated between two risk groups and that were common between VHR/MR, VHR/LR and MR/LR risk groups. (Venny 2.0, (Oliveros, J.C. (2007-2015)***. *LR: low risk, MR: moderate risk, VHR: very high risk.*

***Table 3**: List of proteins that differentiated between different risk groups generated by **Venny 2.0.** (Venny 2.0, (Oliveros, J.C. (2007-2015).*

Stratify VHR/MR (n=24)	Stratify VHR/LR (n=27)	Stratify MR/LR (n=4)	Stratify VHR/MR and VHR/LR (n=22)	Stratify VHR/MR and MR/LR (n=1)	Stratify VHR/LR and MR/LR (n=1)	Common between all risk groups (n=2)
REN	GLO1	TNFRSF11A	MMP_7	IL2_RA	CTSD	LOX_1
FGF_21	MEPE	PAPPA	TR_AP			MMP_9
GDF_15	Notch_3	HO_1	hOSCAR			
IL_18BP	MMP_3	CTSL1	TRAIL_R2			
ICAM_2	TNFRSF10C		PTX3			
PCSK9	LDL_receptor		U_PAR			
SPON1	Gal_3		MMP_12			
IL_1RT1	HAOX1		CEACAM8			
PlGF	MARCO		TNFRSF10A			
TFF3	CD163		AZU1			
OPN	LPL		CNTN1			
LTBR	IL_1ra		PSP_D			
EGFR	MCP_1		NT_pro_BNP			
RARRES2	IDUA		ACE2			
TNF_R1	JAM_A		IL_4RA			
TNFRSF13B	DCN		PRELP			
RETN	IL16		CD4			
IL1RL2	IL_6		PRTN3			
Gal_4	TIE2		PON3			
CHIT1	Gal_9		SCF			
FABP4	OPG		MMP_2			
SRC	IL_17RA		AXL			
vWF	IGFBP_1					
CXCL16	ADAM_TS13					
	CXCL1					
	LEP					
	CD84					

This table represents the number of proteins that differentiated between two risk groups and were unique to those two risk groups (24 proteins for VHR/MR, 27 proteins for VHR/LR and 4 proteins for MR/LR). It also represents the number of proteins that differentiated between two risk groups and were common between two risk groups (22 common proteins that differentiated between VHR/MR and VHR/LR, 1 common protein that differentiated between VHR/LR and MR/LR and 1 common protein that differentiated between MR/LR and VHR/MR). Finally, it represents the 2 proteins that differentiated between two risk groups that were common between VHR/MR, VHR/LR and MR/LR risk groups

6.3.1.3 Properties shared by the proteins that differentiated between different CVD risk groups – Enrichment Analysis

In order to further understand the underlying biological and pathological pathways that the aforementioned proteins share, the online database Enrichr (http://amp.pharm.mssm.edu/Enrichr/) was used to obtain information from the Gene Ontology (biological processes), the Jansen DISEASE, the REACTOME and STRING (version 10.5) online databases and is summarised in **Table 2** in **section III** of the Appendix. Most of the proteins were shown to be involved in coronary artery disease (CAD), cerebrovascular disease, hypertension and arthritis pathways. Some of the notable biological pathways represented by the measured proteins that were induced as a comparison between the different health/disease classes included increased neutrophil degranulation (VHR/MR, VHR/LR and VHR/nonVHR), higher acute and chronic inflammatory response (VHR/MR, VHR/LR and VHR/nonVHR) as well as positive regulation of cell proliferation (VHR/MR).

6.3.1.4 Logistic regression analysis of the proteins that differentiated between different CVD risk groups

To explore whether the proteins that differentiated between two risk groups (Table 3) were effective in predicting cardiovascular risk, a logistic regression analysis was performed. This showed that a model combining the proteins that statistically differentiated between two risk groups was significantly able to predict cohort group membership (Table 4). This suggests that a panel combining multiple protein markers has a greater potential in predicting which individuals are at higher cardiovascular risk.

Once the optimal markers are selected, a specific CVD panel, that could combine a technology close to new proximity extension assay (PEA) technology used in this study (310), could be designed and validated in large cohorts.

However, it is important to consider the threshold in terms of the number of proteins, and possibly risk factors, that need to be added to a certain model in order to achieve an effective risk prediction. It appears that after increasing the number of proteins (Table 4), the improvement in terms of risk prediction is stagnant. Therefore, it is important to find this optimal threshold and choose the proteins that have the greatest contribution to this risk assessment model and validate them in prospective studies. Once selected, and with the help of algorithms computing random forest models (311), those potential proteins could be combined in a multimarker panel, in addition to other CVD risk factors, to possibly assess either CVD risk of first or recurrent events or the response to therapy.

***Table 4:* Logistic regression analysis predicting group membership**.

Cohort	*Protein number*	*p value*	*Percentage of correct prediction by the model*
VHR/MR proteins: Predicting **VHR** (n=208) **vs. MR** (n=28) group membership	49	<0.0001	100%
VHR/LR proteins: Predicting **VHR** (n=208) **vs. LR** (n=61) group membership	52	<0.0001	100%
MR/LR proteins: Predicting **MR** (n=28) **vs. LR** (n=61) group membership	8	0.001	76.4%
VHR/non-VHR proteins: Predicting **VHR** (n=208) **vs. non-VHR** (n=89) group membership	72	<0.0001	100%

LR: low risk; MR: moderate risk; non-VHR: non-very high risk VHR: very high risk. A Binary logistic regression analysis was performed to determine the respective group memberships.

6.3.1.5 Correlation analysis between all measure proteins

To examine whether the measured proteins levels were inter-correlated, Pearson correlation analysis was performed. Refer to **Table 3** in **section III** of the appendix for an example of this correlation. Most of the proteins correlated positively with each other significantly. This highlights the presence of underlying interactions between different biological pathways in CVD and strengthens the idea that a multimarker approach is the way to move forward to improve the assessment of an individual's CVD risk (section 6.3.1.4). Among all the measured proteins, PON3, ADAMTS-13, Chymotripsin C (CTRC) and growth/differentiation factor 2 (GDF-2) appeared to correlate negatively with most of the measured proteins which suggests that they could have an anti-inflammatory and cardioprotective role in CVD. This is proven by some reports indicating that paraoxanases enzymes, such as PON3, are known to be involved in the lipid metabolism (309). Additionally, low levels of ADAMTS-13, which is known to cleave von willbrand factor to control blood clotting, have been linked to adverse outcomes in CVD (312). The role of GDF2 and CTRC in CVD hasn't been intensively studied. In addition, it is important to keep in mind that the development of multimarker panels need to take into account this inter protein correlation since individuals with high levels of one marker will likely have high values of the other.

6.3.1.6 Principle component analysis: Generating protein components to further understand the underlying inflammatory pathways in CVD

In order to investigate whether the list of the aforementioned proteins could be reduced to form protein components, a principal component analysis was carried out. This was to explore whether this analysis could group proteins in terms of the underlying inflammatory pathways that are active in CVD.

The proteins from **section 6.3.1.2** (all proteins from Table 3) that were able to significantly differentiate between different risk groups, formed 9 components where each component captured a direction in the variability of the data. Each component grouped a number of proteins that highly correlated between each other and that had a high contribution to the variability in the component. This is referred to as the protein factor loading. A protein with a highest factor loading within a component is known to contribute the most to the variability of the data within this component. The first generated component is usually known to capture the most variability in the data and the more variability there is, the more components will be generated. The 9 extracted significant components were able to explain 58.8% of the total variability in the data. For each component, a factor score was generated for each participant. This factor score is a linear composite of the optimally-weighted observed proteins in one component and is assigned for each participant.

Table 8 shows the list of proteins in each component (with a threshold for the factor loading set at 0.3) as well as the main biological pathway for each component according to Gene ontology biological pathways and REACTOME online databases. TNFSRF11A had

the highest contribution to component 1 which explained 26.86% of the total variability in the data. On the other hand, HAOX1 had the highest contribution to component 2 which explained 7.04% of the total variability of the data. Table 5 provides the list of proteins and their factor loadings in each component and Figure 7 shows the participants factor scores of components 2, 6 and 8 plotted against component 1.

Results showed that VHR participants had a high factor score for component 1 and 2 whereas non-VHR participants had a low factor score for component 1 and 2. Proteins in component 1 are mainly part of biological pathways involving inflammatory response and proteins in component 2 are known to positively regulate inflammatory response. This translates into a high proinflammatory state in VHR compared to the non-VHR participants (Figure 7a). On the other hand, VHR participants had high factor scores for component 6 which groups proteins involved in the cell interactions at the vascular wall. Therefore, in addition to their high inflammatory state, VHR participants had a higher number of proteins interacting at the vascular wall as opposed to non-VHR participants (Figure 7b). In addition, most of the proteins in component 6 are known to be involved in the initiation or progression of the atherosclerotic lesion (313–316). VHR participants had high factor scores for component 8, which grouped proteins involved in cytokine activation and apoptosis signalling, as opposed to non-VHR participants which would suggest a pro-cytokine activation and a pro-apoptotic state in VHR participants (Figure 7c).

These results demonstrated that a set of potential markers can be grouped in a number of components where each one represents a specific biological pathway in CVD. Evaluating where each individual stands in relation to these components/pathways could provide a better understanding of which pathways are up or downregulated as

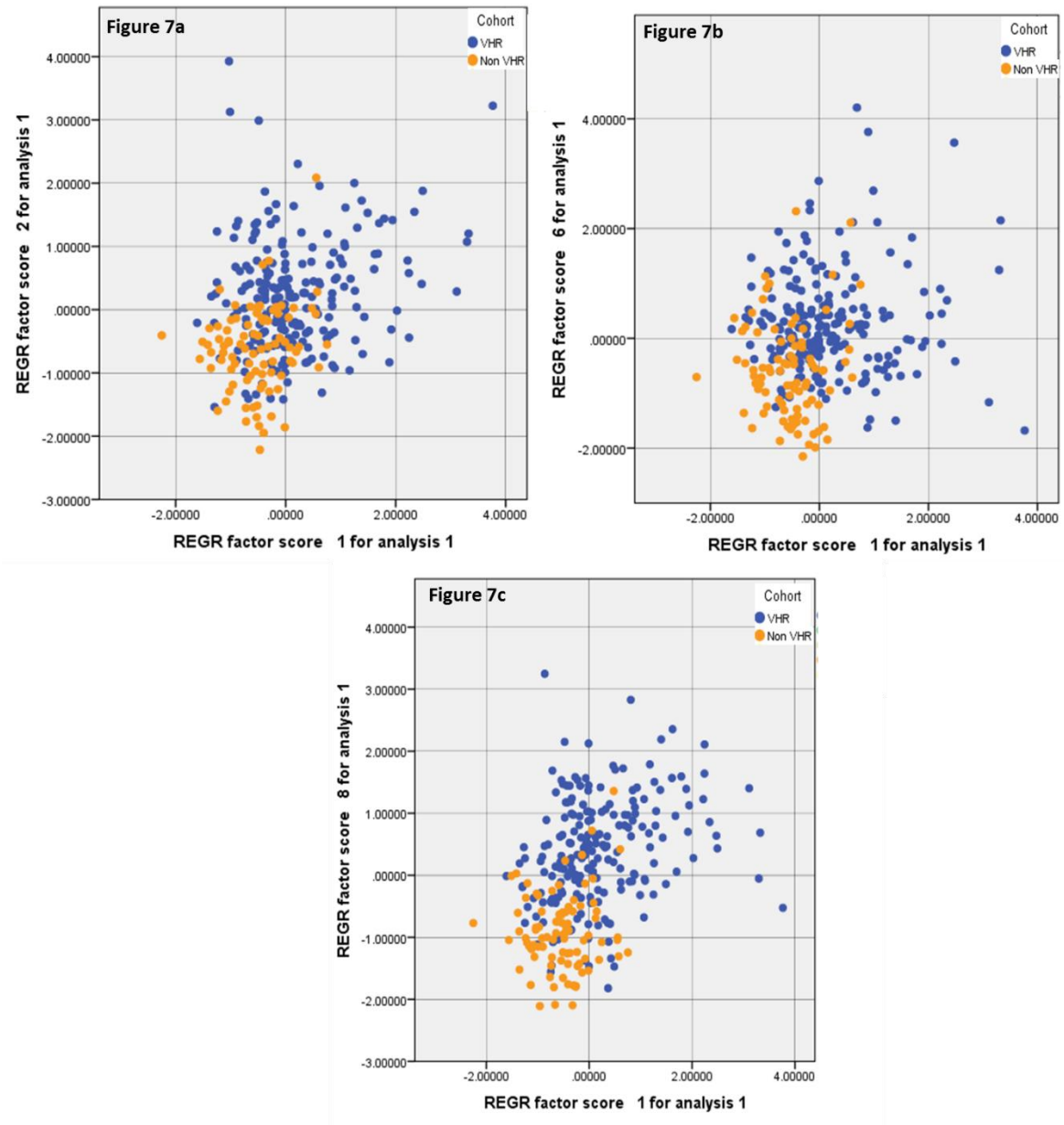
well as the underlying state of CVD. Designing a multimarker panel needs to account for the different inflammatory pathways in CVD where each pathway is represented by its most significant proteins. A PCA could then be used to evaluate which proteins contribute majorly to the pathway in question and help design an optimal panel with the most relevant proteins.

***Table 5:* Principal component analysis of the proteins that significantly differentiated between risk groups**. *Extracted components and main biological pathway for the proteins grouped in each component.*

Component 1		**Component 2**		**Component 3**		**Component 4**		**Component 5**		**Component 6**		**Component 7**		**Component 8**		**Component 9**	
Protein	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*	*Protein*	*Factor loading*
Percentage of total variance explained																	
26.86 %		7.04 %		5.14 %		4.45 %		4.17 %		3.10 %		3.1 %		2.55 %		2.40 %	
TNFRSF11A	.707	IGFBP_1	-.326	PRELP	.351	EGFR	.339	IL_6	.303	CXCL1	-.844	FABP4	-.550	TNF_R1	-.387	EGFR	.340
NT_proBNP	.631	HAOX1	.713	MARCO	.659	DCN	.359	U_PAR	.370	JAM_A	-.794	FGF_21	-.326	LTBR	-.406	GDF_15	-.399
PlGF	.626	ACE2	.688	TIE2	.584	PRELP	.310	IL16	.385	SRC	-.764	PCSK9	-.394	TNFRSF10C	-.333	Gal_9	-.344
EGFR	-.624	CTSD	.587	CD163	.502	SCF	.310	AZU1	.885	CD84	-.726	PTX3	.327	GLO1	.303	TNFRSF13B	-.311
TRAIL_R2	.620	TR_AP	.506	HOSCAR	.456	LPL	.327	LOX_1	.815	GLO1	-.537	LEP	-.779	ADAM_TS13	.631	SPON1	-.344
TNF_R1	.601	SCF	-.477	Gal_9	.449	MMP_2	.722	PRTN3	.807	IDUA	-.319	RARRES2	-.563	MEPE	-.549	PSP_D	-.717
TNFRSF10A	.594	HO_1	.442	TNFRSF13B	.427	CNTN1	.714	CEACAM8	.728			PAPPA	.491	AXL	-.533	MMP_12	-.688
DCN	.585	LPL	-.367	IL16	.397	Notch_3	.712	MMP_9	.727			LDL receptor	-.468	IL_1RT1	-.484	MMP_7	-.547
FABP4	.576	PON3	-.324	IL1RL2	.341	SPON1	.443	RETN	.516			IL_1ra	-.466	ICAM_2	-.451	REN	-.503
TFF3	.574	LDL receptor	.331	AXL	.325	OPG	.407	PTX3	.392			CXCL16	-.375	CXCL16	-.400	CHIT1	-.433
LTBR	.550					PCSK9	.407	TNFRSF10C	.374			Gal_3	-.397	MMP_3	-.335	Gal_3	-.428
IL_4RA	.548					AXL	.419	IL_1ra	.462			Gal_4	-.309	IL_18BP	-.312	Gal_4	-.385
OPN	.545					IL_1RT1	.360	vWF	.361					IL2_RA	-.368	IL2_RA	-.376
PRELP	.537															vWF	-.364
GDF_15	.529															MCP_1	-.321
FGF_21	.506																
IGFBP_1	.481																
IL_6	.458																
CD4	.436																
U_PAR	.396																
Gal_9	.363																
CNTN1	-.371																
SPON1	.310																
OPG	.364																
RETN	.349																
PTX3	.337																
MMP_7	.305																
Biological Pathways (GO biological process or Reactome)																	

Inflammatory response	Positive regulation of inflammatory response	Cholesterol internalisation	Cholesterol homeostasis	Coagulation cascade	Cell surface interactions of the vascular wall	Adipocyte regulation	Cytokine-cytokine receptor interaction and apoptosis signalling	Cytokine signalling in immune system
Jensen diseases								
Arthritis	Coronary artery disease	Skin cancer	Coronary artery disease	Endocarditis	Diabetic	Coronary artery disease	Diabetic nephropathy	Cerebrovascular disease

Principal component analysis was performed using the proteins that were able to significantly differentiate between VHR and non-VHR participants. 9 components were generated with percentage of the total variance explained by each component. Each component groups the proteins that have a factor loading higher than 0.3. The list of proteins within each component was then inputted into Gene Ontology, the Reactome pathways or the Jensen diseases databases in order to identify the main pathway that the proteins of each component belong to.



<u>Figure 7:</u> Principal components based on the proteins that significantly differentiated between risk groups compared between VHR and non-VHR participants. *Component 1 grouped proteins involved in inflammatory response, component 2 grouped proteins implicated in positive regulation of inflammatory response, component 6 grouped proteins associated with the cell surface interactions at the vascular wall and component 8 grouped proteins involved in* involved *in cytokine activation and apoptosis signalling. The plotted values correspond to the participant factor scores for each of component 1, 2, 6 and 8. In Blue are the very high risk (VHR) participants and in orange are the non-VHR participants. Figure 7a: Represents component 1, and 2. VHR participants have a high factor score for component 1 and 2 whereas non-VHR participants have a low factor score for component 1 and 2. Figure 7b: Represents component 1 and 6. VHR participants have a high factor score for component 1 and 6 whereas non-VHR participants have a low factor score for component 1 and 2. Figure 7c: Represents component 1 and 8. VHR participants have a high factor score for component 1 and 8 whereas non-VHR participants have a low factor score for component 1 and 8.*

6.3.2 Biomarkers differentiating between very high risk (VHR) participant subgroups

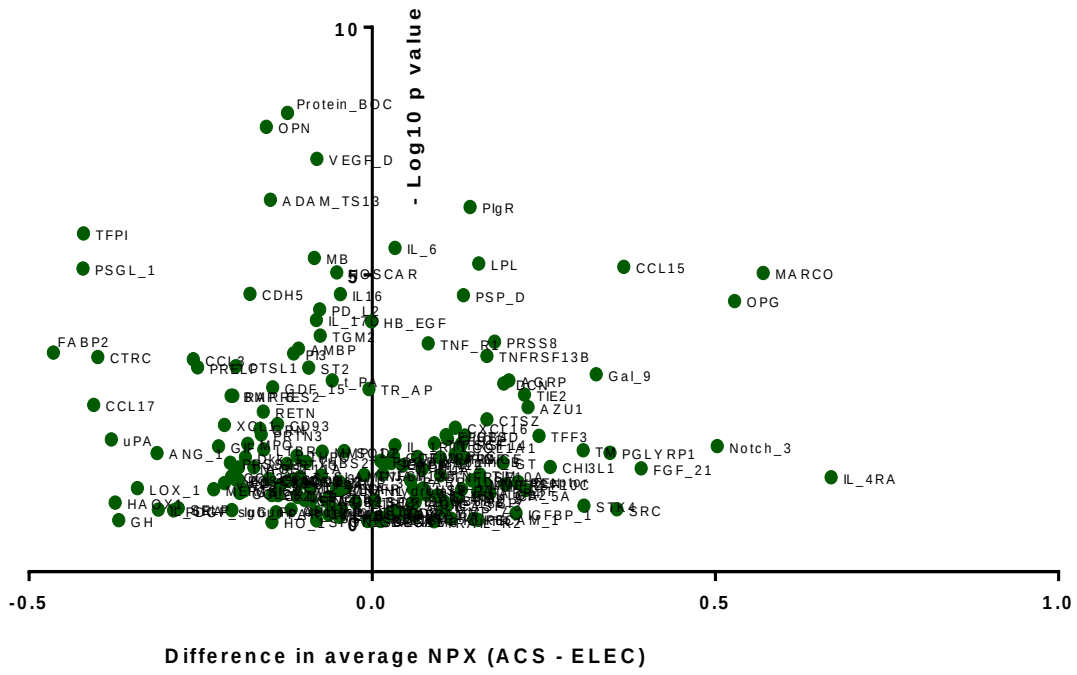
6.3.2.1 Multivariate analysis to select relevant proteins that differentiate between VHR subgroups (Table 9)

In order to explore whether the 184 measured proteins were able to further distinguish between VHR participants, a multivariate analysis (ANCOVA) was conducted. This revealed a set of proteins that characterised VHR participants with an acute cardiovascular event and VHR participants with CVD comorbidities. Refer to **Table 4** in **section III** of the appendix for a full list of significant proteins in each VHR subgroup and p values. Below is the list of proteins that were significantly able to distinguish between VHR subgroup of participants and are described in the context of the current literature (Figure 8, 9, 10, 11, 12, 13, 14).

6.3.2.1.1 ACS-VHR compared to ELEC-VHR participants

Among the 86 proteins that differentiated between acute coronary syndrome (ACS-VHR) and elective percutaneous intervention (ELEC-VHR) participants we cite, Macrophage receptor MARCO (MARCO) ($p<0.0001$), C-C motif chemokine 15 (CCL15) ($p<0.0001$), Osteoprotegerin (OPG) ($p<0.0001$), Polymeric immunoglobulin receptor (PIgR) ($p<0.0001$) which protein levels were higher in ACS-VHR *vs.* ELEC-VHR and Tissue factor pathway inhibitor (TFPI) ($p<0.0001$), P-selectin glycoprotein ligand 1 (PSGL-1) ($p<0.0001$) Fatty acid binding protein 2 (FABP2) ($p<0.001$) and Brother of CDO (BOC) ($p<0.0001$) which protein levels were lower in ACS-VHR *vs.* ELEC-VHR (Figure 8). OPG has been found to be associated with cardiovascular morbidity and mortality after an ACS (317),

whereas TFPI inhibits tissue factor activity and has been shown to attenuate thrombus formation (318). PSGL-1 has been reported to be involved in the recruitment of monocytes to the atherosclerotic lesion site (319) whereas polymorphisms in *FABP2* have been linked to insulin resistance and cardiovascular risk (320). However, there is little evidence of the role of MARCO, CCL15, PIgR and BOC in CVD. It is noteworthy that CRP plasma levels were not significantly different in ACS-VHR compared to ELEC-VHR participants (p=0.942) (Refer to **section 3.3.6** in **Chapter 3)** which suggests that the previously mentioned proteins are good candidates for further investigation as markers of acute cardiovascular events a could prove to be better in further stratifying VHR individuals compared to the present CVD biomarkers.



<u>Figure 8:</u> Volcano plot representing the p value against the difference in mean NPX in protein levels between VHR-ACS and VHR-ELEC participants. *-Log10 of the p value was plotted against the difference between the average of the NPX value (ACS-ELEC). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). (ACS: Acute coronary syndrome participants; ELEC: Elective participants; NPX: normalized protein expression).*

In addition, following the PCA in **section 6.3.1.6,** the factor scores generated for the ACS-VHR and ELEC-VHR were plotted for component 1 and 2 (Figure 9) highlighting the importance of the pro-inflammatory response following an ACS.

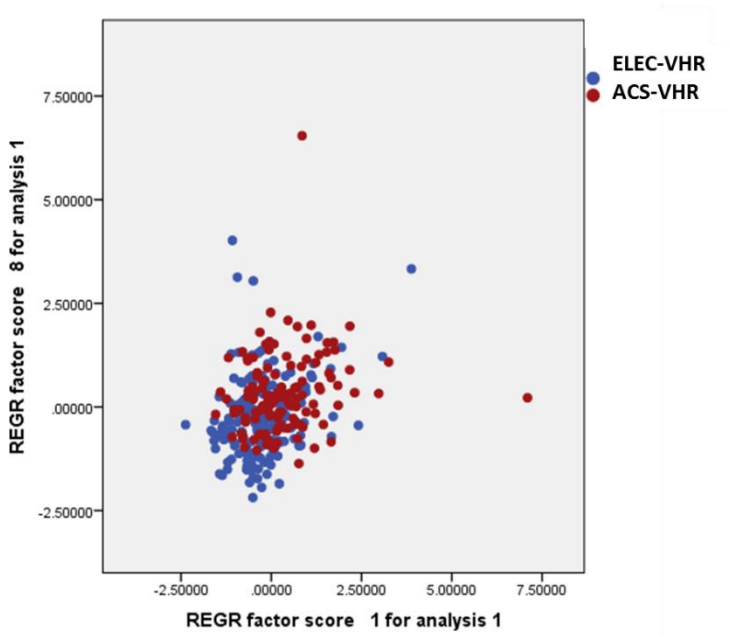


Figure 9: ***Principal components based on the proteins that significantly differentiated between risk groups compared between VHR and non-VHR participants.*** *Component 1 grouped proteins involved in inflammatory response and component 2 grouped proteins implicated in positive regulation of inflammatory response. The plotted values correspond to the participant factor scores for each of component 1 and 2. In Blue are the Elective percutaneous intervention (ELEC-VHR) participants and in red are the acute coronary syndrome (ACS-VHR) participants. ELEC-VHR individuals have a high factor loading for component 1 and 2 compared to the ELEC-VHR individuals.*

6.3.2.1.2 ACS-VHR compared to ELEC-VHR participants with no history of previous CVD

A total of 77 proteins distinguished between ACS-VHR and ELEC-VHR participants with no previous CVD history. These proteins included A disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS-13) ($p<0.0001$), vascular endothelial growth factor D (VEGF-D) ($p<0.0001$), Brother of CDO (BOC) ($p<0.0001$) and programmed cell death 1 ligand 2 (PD-L2) ($p<0.0001$) which protein levels were lower in ACS-VHR

compared to ELEC-VHR participants with no previous CVD. Protein AMBP ($p<0.05$), Osteopontin (OPN) ($p<0.0001$) and Tartrate resistance acid phosphatase 5 (TRAP) ($p<0.0001$) protein levels were higher in ACS-VHR compared to ELEC-VHR participants with no previous CVD (Figure 10). Low protein levels of ADAMTS-13 have been associated with CVD as well as haematological disorders (312). In the present study, ADAMTS-13 levels were lower in ACS-VHR individuals admitted for their primary acute event and are suggested to predict future cardiovascular events (321). OPN has emerged as a potential biomarker and mediator in CVD (322) and in the current study, levels were associated with acute primary events. Protein AMBP is a complex glycoprotein secreted in plasma which role is not fully understood, however, this protein has been recently suggested as a potential biomarker for CVD (323). On the other hand, the role of PD-L2 BOC and TRAP in CVD hasn't been previously outlined. Such proteins should be tested further as biomarkers for first cardiovascular events.

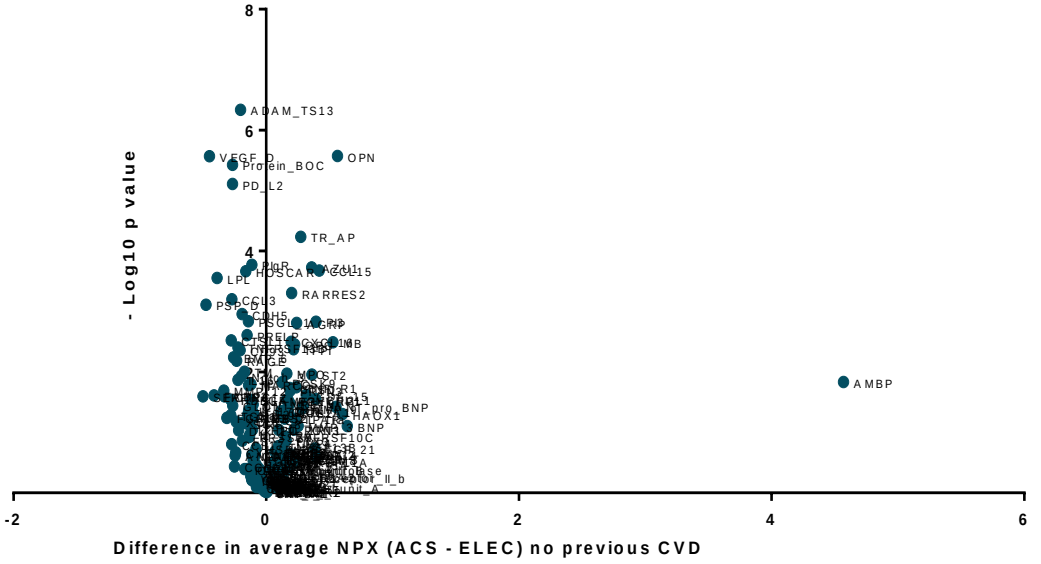


Figure 10: ***Volcano plot representing the p value against the difference in mean NPX in protein levels between VHR-ACS and VHR-ELEC with no previous CVD history.*** *-Log10 of the p value was plotted against the difference between the average of the NPX value (ACS-ELEC). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value ($p<0.0001$). (ACS: Acute*

coronary syndrome participants; CVD: Cardiovascular disease; ELEC: Elective participants; NPX: normalized protein expression).

6.3.2.1.3 ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first cardiovascular event

Among the 40 proteins that differentiated between ACS-VHR participants admitted for recurrent MACE and ACS-VHR participants admitted for their first cardiovascular event we mention Tumour necrosis factor ligand superfamily member 13B (TNFSF13B) ($p<0.01$), Interleukin 18 binding protein (IL18-BP) ($p<0.01$), Tumour necrosis factor receptor superfamily member 11A (TNFRSF11A) ($p<0.01$), Brother of CDO (BOC) ($p<0.01$), Tumour factor alpha receptor 2 (TNFR2) ($p<0.01$) which protein levels were higher in ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first cardiovascular event. Epidermal growth factor receptor (EGFR) ($p<0.05$) protein levels were lower in in ACS-VHR participants admitted for recurrent MACE compared to ACS-VHR participants admitted for their first cardiovascular event (Figure 11). IL18-BP has been reported to have a protective role in a murine model of cardiac ischemia/reperfusion injury (324) and higher plasma levels in individuals with acute secondary events could reflect a protective mechanism of the body in acute phase response. TNFR2 soluble levels have been previously linked to CVD co-morbidities (207) and in the present study, were associated with recurrent events. EGFR activation has been implicated in blood pressure regulation, endothelial dysfunction, atherogenesis, and cardiac remodelling (325). Low plasma levels of EGFR following an acute secondary event, as shown in the current study, could be associated with adverse outcomes. Nevertheless, TNFSF13B, TNFRSF11A, BOC haven't been measured in CVD participants yet. The previously mentioned proteins could be potential markers for secondary cardiovascular events.

Interestingly, it appears that BOC is a marker for ACS events (whether primary or secondary) and hasn't been investigated previously in CVD and the present results are in favour of its role as a potential diagnostic biomarker in CVD.

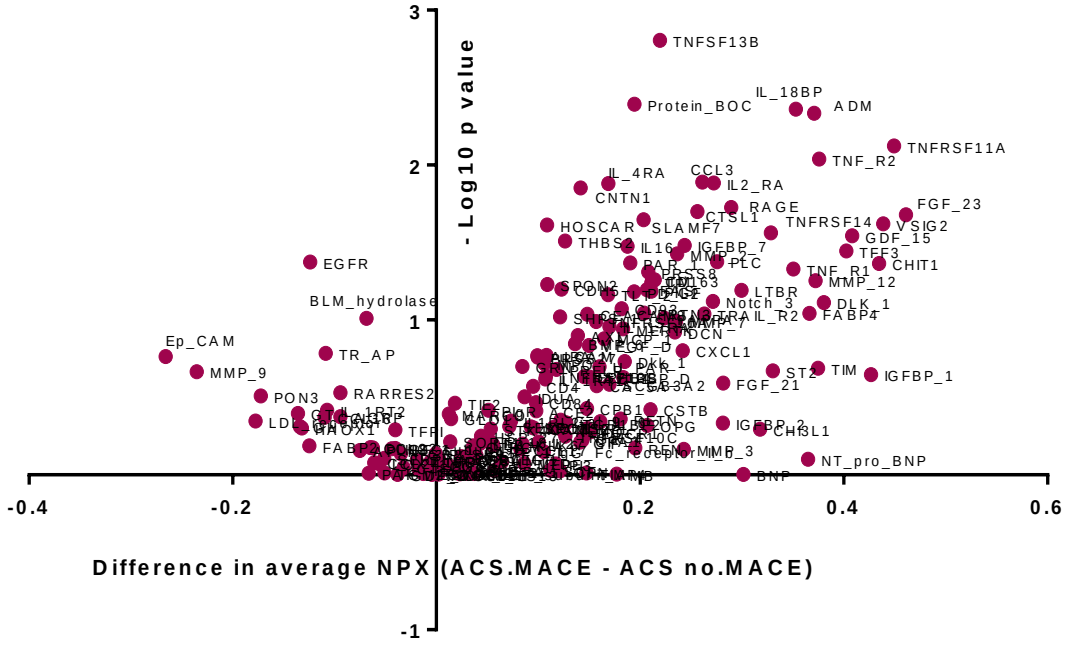


Figure 11: ***Volcano plot representing the p value against the difference in mean NPX in protein levels between ACS participants admitted for MACE and ACS participants admitted for their first cardiovascular event.*** *-Log10 of the p value was plotted against the difference between the average of the NPX value (ACS.MACE-ACS.noMACE). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). (ACS: Acute coronary syndrome participants; MACE: major adverse cardiovascular events; NPX: normalized protein expression).*

6.3.2.1.4 Participants with a previous MI, PCI or CABG

Plasma levels of a total of 77 proteins were higher in VHR participants with previous MI, PCI or CABG compared to those with no previous MI, PCI or CABG. These proteins included V-set and immunoglobulin domain containing protein 2 (VSIG2) (p<0.0001), Brother of CDO (BOC) (p<0.0001) (particularly in those who were acute with a previous MI (p<0.01) or a previous PCI (p<0.05)), Prostasin (PRSS8) (p<0.0001), Interleukin 16 (IL16) (p<0.001) and among those which levels were lower in VHR participants with

previous MI, PCI or CABG compared those with no previous MI, PCI or CABG, we highlight LDL receptor ($p<0.05$) and Tissue factor mi pathway inhibitor (TFPI) ($p<0.05$) (Figure 12). *PRSS8* has been linked to hypertension (326) whereas IL16 has been shown to promote cardiac fibrosis and myocardial stiffening in heart failure participants (327). Moreover, polymorphisms in the *LDLR* gene and LDL receptor protein levels have been associated with atherosclerosis and CVD (328,329). Low circulating plasma levels of LDLR after a statin therapy need to be closely investigated. However, there little evidence of the role of VSIG2 in CVD. Interestingly, CRP plasma levels were not different in VHR participants with a previous MI ($p=0.121$) or a previous PCI ($p=0.887$) (n=294) which indicates that the previously mentioned proteins could be promising biomarkers for treatment response in CVD.

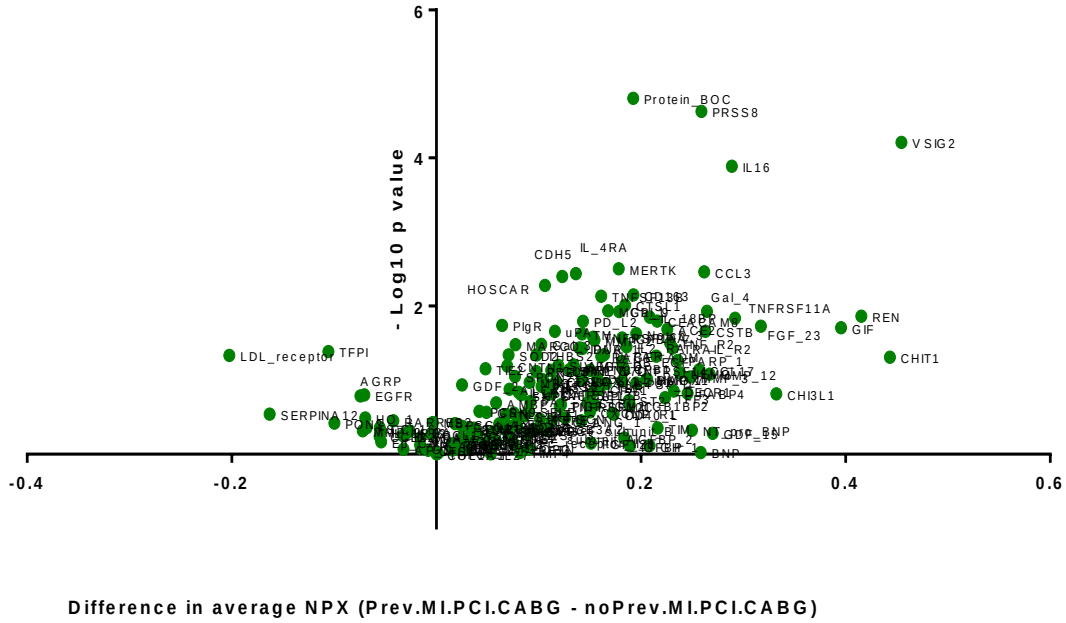


Figure 12: ***Volcano plot representing the p value against the difference in mean NPX in protein levels between VHR participants with previous MI, PCI or CABG and VHR participants with no previous MI, PCI or CABG.*** *-Log10 of the p value was plotted against the difference between the average of the NPX value (Previous MI, PCI, CABG – no previous MI, PCI, CABG). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value ($p<0.0001$). (CABG: coronary artery bypass grafting, MI: myocardial infarction, PCI: percutaneous coronary intervention; NPX: normalized protein expression).*

6.3.2.1.5 VHR participants with diabetes

A total of 83 proteins were able to differentiate between VHR diabetic participants compared to VHR non-diabetic participants. Among those proteins, those with higher levels in the VHR diabetic participants included Kidney injury molecule 1 (previously TIM now KIM1) ($p<0.0001$), Interleukin-1 receptor type 1 (IL-1RT1) ($p<0.0001$), Galectin 3 (Gal-3) ($p<0.0001$), Renin (REN) ($p<0.0001$), V-set and immunoglobulin domain containing protein 2 (VSIG2) ($p<0.0001$), Fatty acid binding protein adipocyte (FABP4) ($p<0.001$) and Gastric intrinsic factor (GIF) ($p<0.001$). Protein which levels were lower in VHR diabetic *vs.* VHR non-diabetic participants included Paraoxonoase 3 (PON3) ($p<0.01$) and Heme oxygenase 1 (HO-1) ($p<0.05$) (Figure 13). KIM1 protein levels have been linked to renal disease in diabetic participants (330) and have also been associated with cardiovascular risk (331). *IL1RT1* gene has been shown to have a role in diabetes (332) and to regulate infarct healing and cardiac remodelling (333). Gal-3 and FABP4 have been associated with the development of CVD (334,335) and diabetes (336,337), whereas REN has been shown to predict cardiovascular mortality (307) and diabetes (338) in participants with hypertension. PON3 is an antioxidant molecule which has been shown to prevent atherosclerosis nevertheless, its role in diabetes is less studied (339). On the other hand, HO-1 has potent anti-inflammatory and anti-oxidative functions in CVD and diabetes (340,341) and higher levels could be associated with a protective effect in VHR indivduals with diabetes. However, the role of VSIG2 and GIF in CVD and diabetes is less clear. Such proteins could be markers for VHR patients at high risk of developing diabetes or *vice versa* and could also prove to be promising new therapeutical targets.

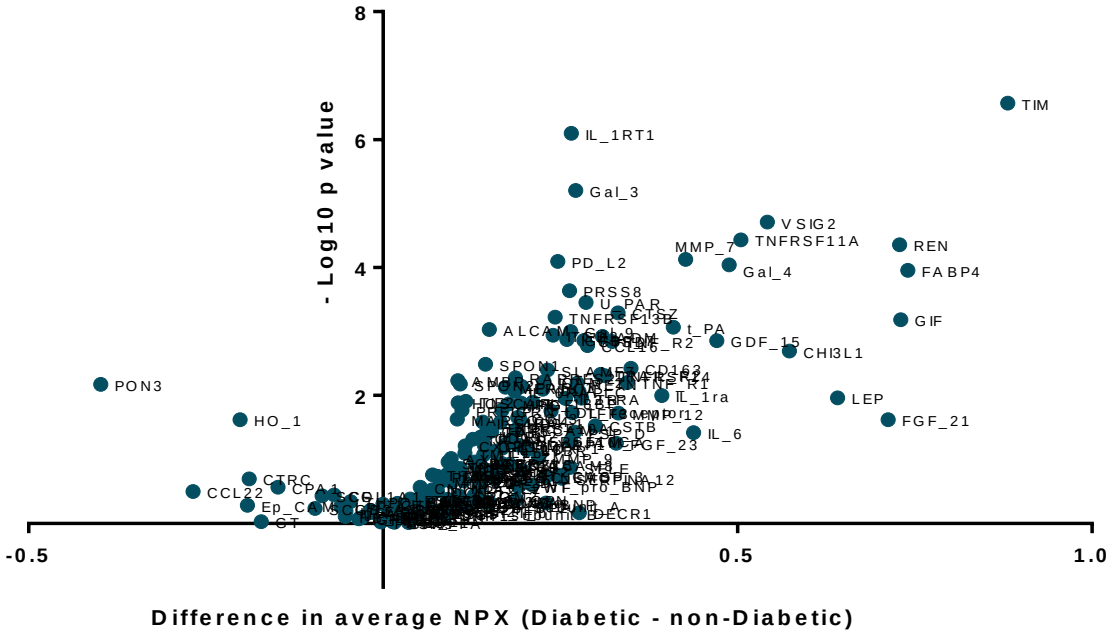


Figure 13: ***Volcano plot representing the p value against the difference in mean NPX in protein levels between VHR diabetic participants and VHR non-diabetic participants.*** *- Log10 of the p value was plotted against the difference between the average of the NPX value (Diabetic – non-diabetic). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). (NPX: normalized protein expression).*

6.3.2.1.6 VHR participants with heart failure

A total of 71 protein were able to distinguish between VHR participants with heart failure compared to the VHR participants with no heart failure. Proteins which levels were higher in VHR participants with heart failure included TNF related apoptosis-inducing ligand receptor 2 (TRAIL-R2) (p<0.0001), Fibroblast growth factor 21 (FGF-21) (p<0.001), Adrenomedullin (ADM) (p<0.0001) and Tumour necrosis factor receptor superfamily member 11A (TNFRSF11A) (p<0.0001). Among the proteins which levels were lower in VHR participants with heart failure compared to VHR participants with no heart failure we underline, Chymotrypsin C (CTRC) (p<0.001) and Proto-oncogene tyrosine-protein kinase (SRC) (p<0.05) (Figure 14). FGF-21 has been reported to be associated with cardiovascular risk (342) and circulating FGF-21 levels correlated with

diastolic dysfunction in participants with heart failure (343). In addition, ADM, which is known to regulate vasodilatation and nitric oxide production, has been suggested to play a protective role in CVD and heart failure (344). However, the role of TNFRS11A and CTRC in CVD and heart failure is less clear. Since heart failure is a major complication after an ACS (345), such proteins could be markers for VHR patients at high risk of developing heart failure and could also prove to be promising new therapeutical targets.

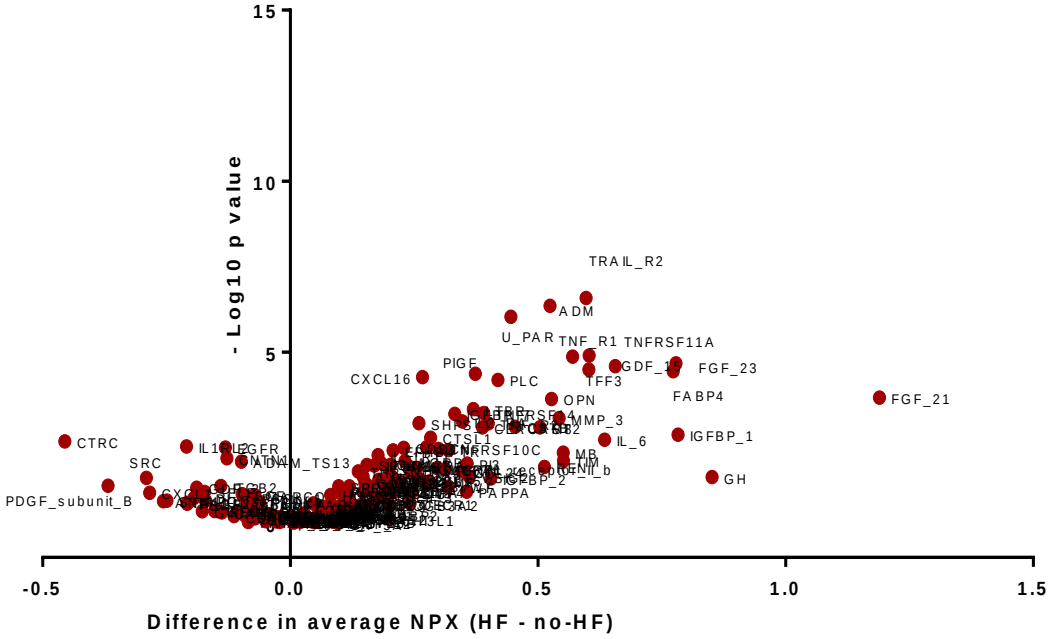


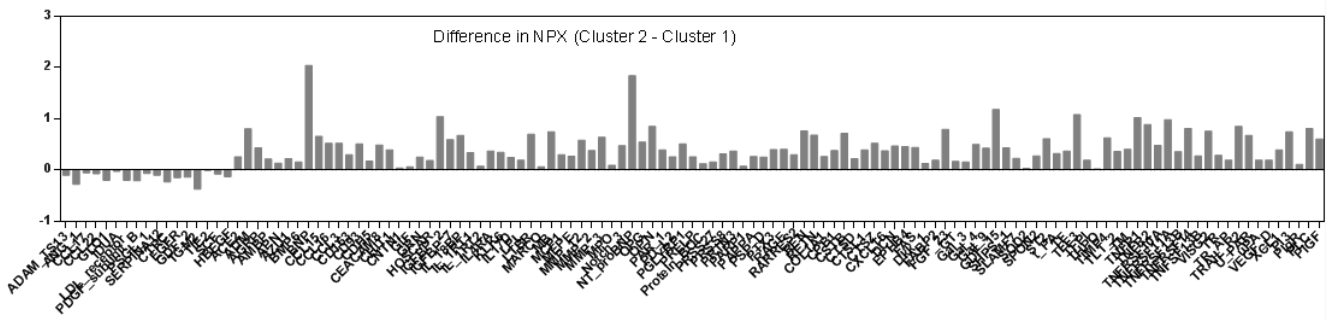
Figure 14: ***Volcano plot representing the p value against the difference in mean NPX in protein levels between VHR participants with heart failure and VHR participants with no heart failure*** *-Log10 of the p value was plotted against the difference between the average of the NPX value (HF- no HF). Proteins to the far left or right side of the graph have a significant fold in change (whether higher or lower) with a very significant p value (p<0.0001). (HF: heart failure; NPX: normalized protein expression*

6.3.2.2 Cluster analysis to further stratify the VHR cohort using proteins associated with MACE risk

To explore whether the significant proteins associated with MACE events (proteins from group 1, 2 and 4 in **Table 4** in **section III** of the appendix) could further stratify VHR individuals based on their plasma levels, a cluster analysis (two-way cluster analysis) was performed. Results showed that the VHR cohort formed two clusters with cluster 2 (n=59) grouping participants who had higher levels of most of the inflammatory markers compared to cluster 1 (n=149) (Figure 15) (Refer to **Table 5** in **section III** of the appendix for the protein levels in each cluster). Cluster 2 grouped older participants, participants who were likely to be admitted for an ACS and for recurrent MACE. Cluster 2 also grouped participants who were more likely to be diabetic, to have had a previous MI or PCI, and to be on CVD medication. The proteins that contributed majorly to the cluster classification were: Perlecan (PLC), Trefoil factor 3 (TFF3), Tumour necrosis factor alpha receptor 1 (TNFR1) and 2 (TNFR2), Growth/differentiation factor 15 (GDF15), Adrenomedullin (ADM), TNF Receptor Superfamily Member 14 (TNFRSF14), Urokinase receptor (UPAR) and Lymphotoxin Beta Receptor (LTBR) which were all higher in cluster 2 ($p<0.0001$). Among these significant proteins, PLC has an essential function in skeletal muscle and cardiovascular development (346) with a promising role in preventing stent-restenosis since it inhibits smooth muscle cells activity and thrombosis while enhancing endothelial cell proliferation (347). ADM has been proposed to be a biomarker of prognosis and survival in participants with coronary artery disease or heart failure (344). In addition, TNFRSF14 is a contributor in atherogenesis by activating proinflammatory cytokines and matrix metalloproteinases (348). UPAR has been intensively studied in chronic kidney disease (CKD) and has been suggested as a biomarker for CVD in participants with CKD (349). Moreover, LTBR appears to play a role in macrophage-

driven inflammation in atherosclerotic lesions (350). However, the role of TFF3 in CVD is less studied.

These results highlight and support the role of such proteins in further stratifying individuals with an established CVD based on protein levels alone. Such proteins require further prospective studies to assess their role in clinical practice. This data also brings to attention the importance of cluster analysis in patient stratification. Therefore, based on differences in plasma levels of significant protein, individuals with a critical pro-inflammatory state and a severe CVD history can form a distinctive group within the global VHR cohort. This highlights the need to define protein level thresholds within the VHR cohort. Individuals with very high levels of inflammatory markers could require intensive medical management and close follow-up.



<u>Figure 15:</u> Difference in proteins means (NPX) between cluster 2 and cluster 1 subgroups within the VHR cohort. *A two way cluster analysis was performed which grouped participants into two clusters where particpiants belonging to same cluster share common charactersitics. Each cluster was defined by a biomarker signature.*

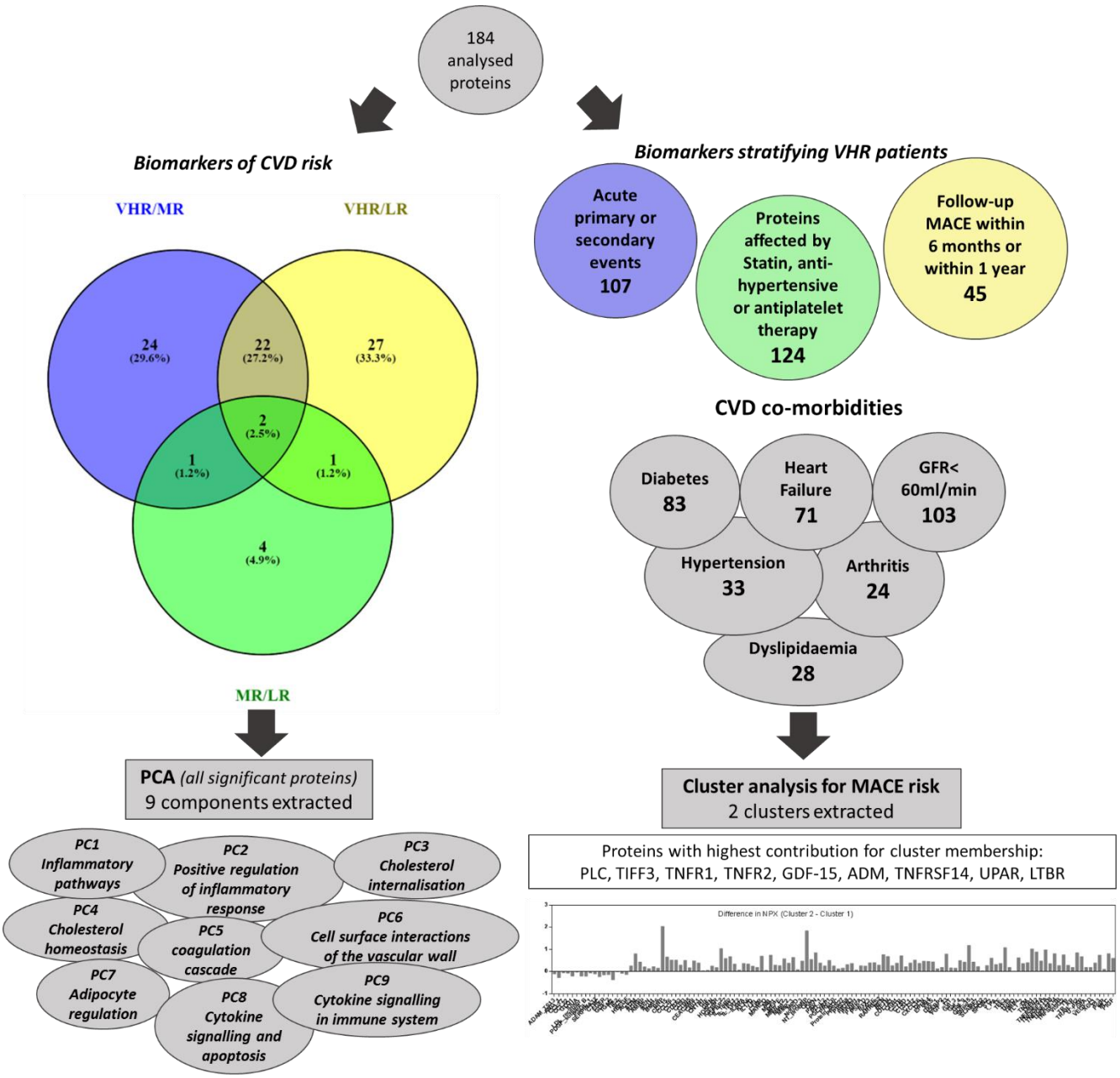


Figure 16: ***Summary diagram - Proteomic approach for the selection of potential CVD biomarkers of cardiovascular risk and MACE risk.*** *CVD: cardiovascular disease; GFR: glomerular filtration rate; LR: low risk, MACE: major adverse cardiovascular events; MR: moderate risk, PC: principal component; PCA: principal component analysis; VHR: very high risk.*

6.3.3 Multimarker approach in biomarker research and development

In biomarker research, the consensus nowadays has shifted towards a multimarker approach due to CVD complexity and since it is now better understood that one single biomarker is unlikely to capture the individual's predisposition to disease development. Previous studies have investigated the role of some of the proteins that were highlighted in this study in CVD. However, each study focused on one single biomarker alone linking it to a specific biological pathway, a specific clinical outcome or a specific CVD co-morbidity. This study highlighted the importance in using multimarker panels in assessing CVD risk and further stratifying individuals at high risk of MACE. However, such panels need to be optimised to select the proteins that are the most representative of the different CVD inflammatory pathways. In addition, the number of optimal proteins needed in order to achieve the most effective risk prediction needs to be addressed. Such panels could be useful in CVD risk assessment, but also in predicting cardiac events and treatment response (137). New models and algorithms combining such proteins in a tool that assesses CVD risk need to be developed and taken forward into clinical assessment.

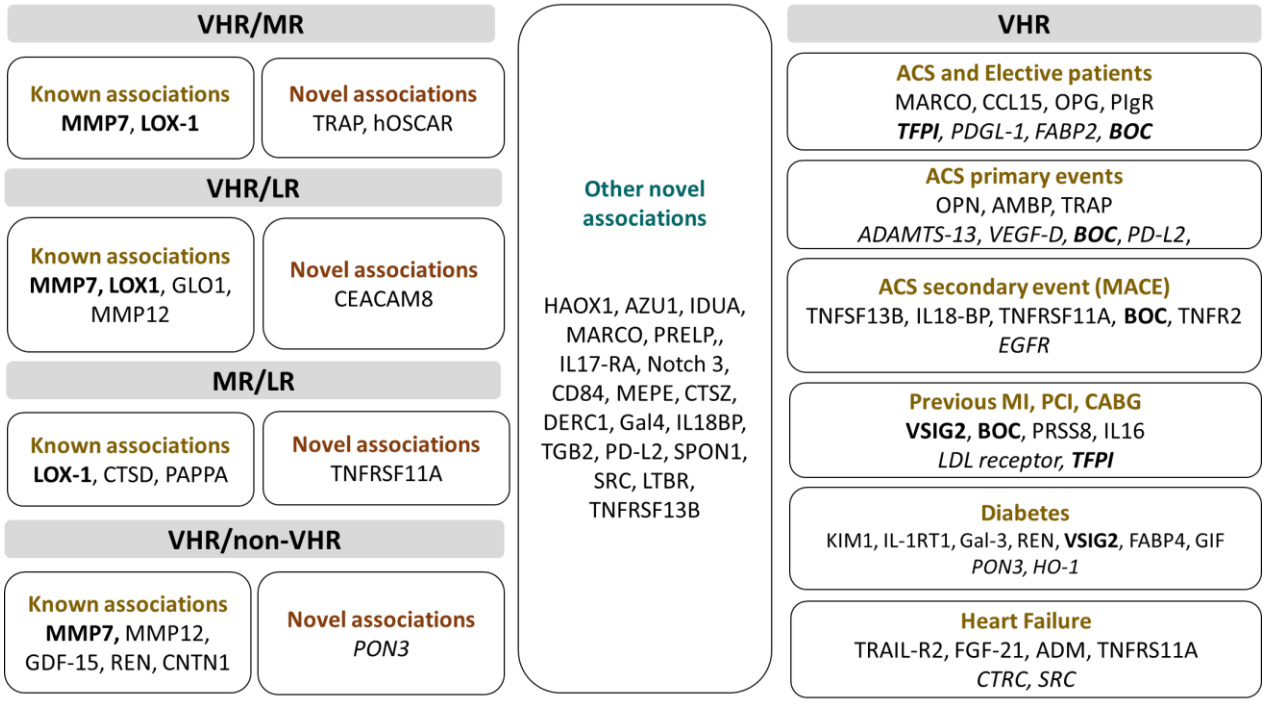


Figure 17: ***Summary diagram of a proteomic analysis: Potential proteins for CVD risk stratification.*** *Proteins in bold were able to differentiate between more than two cohorts. Proteins in italic were lower in the two compared subgroups. ACS: acute coronary syndrome; CABG: coronary artery bypass grafting; LR: low risk, MACE: major adverse cardiovascular events; MI: myocardial infarction MR: moderate risk, PCI: percutaneous coronary intervention; VHR: very high risk.*

6.3.4 Strengths and limitations

Refer to **section 3.4.3** in **Chapter 3.**

In relation to the natriuretic peptides (NP) that were measure in this study; BNP and NT-pro BNP protein levels were below the limit of detection of the assay in many participants, therefore, they were excluded from the analysis. However, the analysis of the available results showed a strong association with cardiovascular risk.

6.4 Conclusion

The present investigation has several potential clinical implications. First, it highlights candidate biomarkers that can stratify individuals at various levels of CVD risk. Second, it underlines specific markers that are able to further differentiate between very high

risk individuals in relation to their CVD history (whether they have a primary or a secondary event/MACE) or according to their co-morbidities (diabetes, heart failure, chronic kidney diseases). This study also points out the importance of a multimarker approach in reflecting the underlying inflammatory pathways in CVD and in establishing a unique proteomic disease signature for each individual.

Chapter 7

Biomarkers predicting MACE risk within one year of admission in individuals at very high cardiovascular risk

Abstract

Background: Traditional cardiovascular disease (CVD) risk factors are effective in primary prevention but less effective when it comes to secondary prevention and the prediction of major adverse cardiovascular events (MACE). Additionally, the processes and biological pathways behind recurrent heart attacks, stent restenosis, persistent angina, drug resistance and sudden deaths are not clear. In this present study, we explore the association between MACE risk and a number of proteins involved in the immune response, plaque formation and thrombosis in participants with established CVD.

Methods: Participants were recruited from the cardiac catheterisation laboratory or by email advertisement. Participants were all at very high risk (VHR) with a 10-year risk SCORE ≥10% risk of fatal CVD. TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels were measured by ELISA. INF-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNFα plasma protein levels were measured using MSD® MULTI-SPOT Assay System. *TACE* mRNA levels were measured by quantitative real-time PCR. CVDII and CVDIII panels were measured in the plasma by multiplex proximity extension assays (PEA) by Olink® proteomics.

Results: At total of 229 VHR participants were recruited. TNFR1 (HR; 5.474; $p<0.05$), IL-6 (HR: 10.29; $p<0.001$), ST2 (HR: 4.37; $p<0.01$), IL4RA (HR:7.51; $p<0.01$), IL1RT1 (HR:11.89; $p<0.01$), IL1RT2 (HR: 7.242; $p<0.01$), PDL2 (HR:11.34; $p<0.01$), ADM (HR:3.37; $p<0.01$), THBS2 (HR:13.64; $p<0.01$), CCL16 (HR:5.60; $p<0.05$), RARRES2 (HR:20.30; $p<0.05$), GDF15 (HR:2.17; $p<0.05$), PI3 (HR:1.78; $p<0.05$) and SPON1 (HR:7.96; $p<0.05$) plasma protein levels were higher on admission in VHR participants who developed MACE within a year of admission compared to those with no readmission within this year whereas, CCL22 (HR: 0.249; $p<0.01$), ADAM_TS13 (HR:0.022; $p<0.05$) and IDUA

(HR:0.324; $p<0.05$) plasma protein levels were lower on admission in participants who developed MACE within a year of admission compared to those with no readmission within this year.

Conclusion: In conclusion, the present investigation underlines previously reported and novel associations between several inflammatory proteins and MACE risk. These CVD markers should be taken forward into biomarker development for MACE risk assessment. If adopted in clinical practice, such biomarkers have the potential to reduce the number of CVD participants who develop recurrent events by identifying them earlier and providing them with a better medical and clinical management.

7.1 Introduction

Biomarkers and risk factors associated with cardiovascular risk and the development of a first cardiovascular event in patients with no previous cardiac history have been intensively studied (81). Traditional cardiovascular risk factors such as high cholesterol levels, hypertension and smoking are relevant in primary prevention. In addition, the inflammatory and immune pathways behind the development of atherosclerosis and eventually first cardiovascular events are well established (351).

Nevertheless, biomarkers and risk factors associated with the development of recurrent major adverse cardiovascular events (MACE) need better understanding as the processes and biological pathways behind recurrent heart attacks, stent restenosis, persistent angina, drug resistance and sudden deaths are less clear. The management of coronary artery disease (CAD), one of the major causes of cardiovascular disease (CVD), with revascularisation strategies and aggressive medical therapy is proving to be insufficient as the occurrence of MACE remains very high (18). At present, there are no available test that could reveal which person is more likely to develop MACE. Risk scores, such as the GRACE and the TIMI scores (106) are in place to evaluate if a patient admitted with a first cardiovascular event is likely to develop a secondary event based on a number of characteristics upon admission. However, these risk scores present many limitations and are not widely used in clinical practice (106) and therefore need to be improved to include different factors and markers that are associated with recurrent events (352).

We undertook the present investigation to evaluate the association between several markers and MACE risk in individuals with an established CVD using two of the most common methods in biomarker discovery. First, we used a deductive method where we

looked at the pathway involving tumour necrosis factor alpha (TNFα), its converting enzyme (TACE) as well as both of its receptors 1 (TNFR1) and 2 (TNFR2) and endogenous inhibitor (TIMP3). This was to evaluate whether MACE can be predicted by a panel of biomarkers that interact with each other in a well-studied biological pathway. Second, we followed an inductive proteomic method to evaluate the association between MACE risk and a large set of inflammatory proteins known to be involved in immunity, atherosclerosis, plaque rupture and thrombosis.

7.2 Participants and Methods

Refer to **section 2.1** in **Chapter 2** for a detailed description of the participant recruitment process, the risk score used and the methods for blood processing. Participants were all at very high risk (VHR) as per the European low risk SCORE chart which is used to determine the 10-year risk of fatal CVD in low risk regions of Europe by gender, age, systolic blood pressure, total cholesterol and smoking status for all recruited individuals who were from Ireland.

7.2.1 Demographic information

Refer to section **3.2.1 in Chapter 3** for a detailed description of the demographic information that was collected in this study.

7.2.2 MACE follow up

Major adverse cardiovascular events (MACE) were regarded as any deaths, admissions for acute coronary syndrome (ACS) or stroke and were recorded for all the VHR participants within one year following admission using the Northern Ireland Electronic

Care Record (NIECR). The one year follow-up period was then subdivided into MACE within 6 months and MACE after 6 months.

7.2.4 Measurement of TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels

Refer to **section 2.6.1, 2.7.1, 2.8.1, 2.9.1 in Chapter 2.**

7.2.5 Measurement of *TACE* gene expression

Refer to **section 2.11, 2.12 and 2.13 in Chapter 2.**

7.2.6 Measurement of TNFα, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IL-13 plasma protein levels

Refer to **section 2.10 in Chapter 2.**

7.2.7 Analysis of O-link Cardiovascular panel II and III (O-link Proteomics®) in plasma samples

Refer to **section 2.14 in Chapter 2.**

7.2.8 Statistical methods

Statistical analysis was carried out as described in **section 3.2.7** in **Chapter 3**

In addition, a cox regression model was used to calculate the hazard ratios for the measured biomarkers and the development of MACE over time. Logarithmic values were used for biomarker levels. Each increase in one unit corresponds to the calculated hazard ratio. Statistical significance was defined as values of $p<0.05$ (two-tailed).

7.3 Results

7.3.1 Participant recruitment

Refer to section **3.3.1 in Chapter 3**.

Table 1 and 2 provide the full descriptive statistics of the population. Follow-up MACE were recorded within one year of admission and were divided into events within 6 months and after 6 months of admission. MACE were defined as any deaths, admissions for acute coronary syndrome (ACS) and stroke. After a follow-up duration of 1 year and among 229 VHR participants, 6.98 % of participants developed MACE in total; 2.18% developed MACE within 6 months and 4.80% developed MACE after 6 months.

Table 1: ***Baseline demographics and lifestyle characteristics of the VHR cohort.***

Demographic and history	**Number of participants (n)**	**Percentage of participants (%)**
Gender		
Male	180	78.6
Female	49	21.4
Diabetes mellitus		
Present	44	19.2
Absent	185	80.8
Heart Failure		
Present	36	15.7
Absent	193	84.3
Renal function		
GFR < 60	45	20
GFR > 60	180	80
Hypertension		
Present	128	58.9
Absent	101	44.1
Dyslipidaemia		
Present	134	58.5
Absent	95	41.5
Arthritis		
Present	35	15.3
Absent	194	84.7
Depression		
Present	43	18.8
Absent	186	81.2
Present or previous cancer		
Present	27	11.8
Absent	202	88.2

Smoking		
none	66	30.3
ex-smoker	110	50.4
current	42	19.3
Pharmacotherapy (n; %)		
Statin therapy		
Present	165	72.7
Absent	62	27.3
Antiplatelet therapy		
Present	154	67.8
Absent	73	32.1
Antihypertensive therapy		
Present	172	76.8
Absent	55	24.2
Anti-anginal therapy		
Present	88	38.8
Absent	139	61.2
Diuretic therapy		
Present	56	24.7
Absent	171	75.3
Drug naïve		
Present	54	23.7
Absent	174	76.3
Clinical Variables (n; %)		
VHR-ACS	127	55.5
VHR-ELEC	102	44.5
Previous MI		
Present	73	31.9
Absent	156	68.1
Previous PCI		
Present	73	31.9
Absent	56	68.1
Previous CABG		
Present	22	9.6
Absent	207	90.4
Previous MI, PCI or CABG		
Present	100	43.7
Absent	129	56.3
Diagnosis upon admission		
Stable Angina	85	37.1
Unstable Angina	21	9.2
NSTEMI < 1 week	77	33.6
STEMI < 1 week	7	3.0
Other	39	17.03
ACS participants admitted for MACE		
present	45	35.4
absent	82	64.6
Participants with no previous MI, PCI or CABG		
ACS-VHR	84	65.1
ELEC-VHR	45	34.9

ACS: acute coronary syndrome participants; CABG: Coronary artery bypass surgery; ELEC: elective participants; GFR: Glomerular filtration rate; MACE: major adverse cardiovascular events MI: Myocardial Infarction; NSTEMI: non-ST elevation myocardial infarction; PCI: percutaneous coronary intervention; STEMI: ST elevation myocardial infarction; VHR: very high risk.

***Table 2:* Descriptive statistics of VHR participants who developed MACE within a year.**

	No MACE (n=213)		MACE (n=16)		*p value*
	mean	*Std. deviation*	*mean*	*Std. deviation*	
Age (years)	65	11	74	7	0.001
Cholesterol levels (mmol/L)	4.2	1.3	3.5	0.8	0.022
Systolic BP (mmHg)	131	23	134	30	0.61
BMI (Kg/m^2)	29.1	5.8	30.3	11.7	0.469
	n; %		*n; %*		
Diabetes	34; 18.3		5; 31.3		0.171
Heart Failure	29; 13.6		7; 43.8		0.005
GFR<60	38; 18.2		7; 43.8		0.022
On medication	160; 75.5		14; 87.5		0.222
ACS participants	117; 54.9		10; 62.5		0.375
Statin therapy	152; 72.0		13; 81.3		0.318
Antiplatelet therapy	142; 67.3		12; 75		0.37
Antihypertensive therapy	157; 74.4		15; 93.8		0.065
Previous MI, PCI or CABG	93; 43.7		7; 43.8		0.569

ACS: acute coronary syndrome participants; BMI: body mass index; BP: blood pressure; CABG: Coronary artery bypass surgery; GFR: Glomerular filtration rate; MACE: major adverse cardiovascular events; MI: Myocardial Infarction; NSTEMI: non-ST elevation myocardial infarction; PCI: percutaneous coronary intervention; STEMI: ST elevation myocardial infarction; VHR: very high risk. A Student t-test was performed to compare means of continuous variables and a Pearson chi square test was performed to compare percentages of categorical variables.

7.3.2 Biomarkers associated with MACE within 1 year of admission

In order to investigate whether the measured proteins were associated with MACE occurrence, VHR participants who developed MACE within 6 months or after 6 months were compared to those who were not readmitted within the year. Results showed that TNFR1 plasma protein levels were higher upon admission in participants who developed MACE within a year ($p<0.05$) and levels tended to be higher in participants who developed MACE after 6 months with a p value near significance ($p=0.061$). On the other hand, IL-6 plasma protein levels were higher upon admission in participants who

developed MACE within a year ($p<0.05$) and were particularly higher among the participants who developed MACE within 6 months ($p<0.05$) (Table 1). Among the VHR individuals who developed MACE over time, 62.5% (n=10) were ACS-VHR and 37.5% (n=6) were ELEC-VHR. In the Olink analysis, TNFR1 and IL-6 plasma protein levels were also higher in VHR participants who developed MACE within a year of admission ($p<0.001$ and $p<0.05$). Refer to **Table 7** in **section III** of the Appendix for the full list of proteins and p values.

Among the proteins tested as part of the CVDII and CVDIII Olink® proteomic panel, Retinoic acid receptor responder protein 2 (RARRES2) ($p<0.001$), C-C motif chemokine 16 (CCL16) ($p<0.001$), Interleukin-1 receptor type 1 (IL1RT1) ($p<0.001$), Trefoil factor 3 (TFF3) ($p<0.01$), Adrenomedullin (ADM) ($p<0.01$), C-X-C chemokine motif 16 (CXCL16) ($p<0.004$), Transferrin receptor protein 1 (TR) ($p<0.005$), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2) ($p<0.006$), Matrix metalloproteinase 3 (MMP3) ($p<0.007$), Lymphotoxin-beta receptor (LTBR) ($p<0.008$), Tumour necrosis factor receptor superfamily member 14 (TNFRSF14) ($p<0.008$), Programmed cell death 1 ligand 2 (PDL2) ($p<0.008$), Growth/differentiation factor 15 (GDF15) ($p<0.01$), Ephrin type-B receptor 4 (EPHB4) ($p<0.05$), Interleukin-2 receptor subunit alpha (IL2RA) ($p<0.05$), Tyrosine-protein phosphatase non-receptor type substrate 1 (SHPS1) ($p<0.05$), Agouti related protein (AGRP) ($p<0.05$), Interleukin 1 receptor 2 (IL1RT2) ($p<0.05$), Kallikrein-6 (KLK6) ($p<0.05$), Thrombospondin 2 (THBS2) ($p<0.05$), Interleukin-4 receptor subunit alpha (IL4RA) ($p<0.05$), Elafin (PI3) ($p<0.05$), Perlecan (PLC) ($p<0.05$), Renin (REN) ($p<0.05$), V-set and immunoglobulin domain-containing protein 2 (VSIG2) ($p<0.05$), Insulin-like growth factor-binding protein 2 (IGFBP2) ($p<0.05$), Urokinase plasminogen activator surface receptor (UPAR) ($p<0.05$), Fibroblast growth factor 23 (FGF23) ($p<0.05$), Protein

Alpha-1-Microglobulin/Bikunin Precursor (AMBP) ($p<0.05$), Cathepsin D (CTSD) ($p<0.05$), Receptor for advanced glycosylation end products (RAGE) ($p<0.05$), Tumour necrosis factor alpha receptor 2 (TNFR2) ($p<0.05$), Granulins (GRN) ($p<0.05$), Complement component C1q receptor (CD93) ($p<0.05$), Proteinase activated receptor 1 (PAR1) ($p<0.05$), Spondin 1 (SPON1) ($p<0.05$), Interleukin 1 receptor-like 1 (ST2) ($p<0.05$), Fibroblast growth factor 21 (FGF21) ($p<0.05$), Carboxypeptidase 1 (CPA1) ($p<0.05$), C-C motif chemokine 15 (CCL15) ($p<0.05$) and Insulin like growth factor binding protein 7 (IGFBP7) ($p<0.05$) protein levels were higher in VHR participants who developed MACE within a year of admission. On the other hand, Plasma A disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS13) ($p<0.01$), Alpha-L-iduronidase (IDUA) ($p<0.05$), C-C motif chemokine 22 (CCL22) ($p<0.05$) and Interleukin 1 receptor like 2 (IL1RL2) ($p<0.05$) protein levels were lower in participants who developed MACE within a year of admission compared to those with no readmission within a year.

Plasma protein levels that were particularly higher in VHR participants who developed MACE within 6 months were TR ($p<0.05$), TNFRSF14 ($p<0.05$), GDF15 ($p<0.05$), AGRP ($p<0.05$), THBS2 ($p<0.05$), PI3 ($p<0.05$), IL-6 ($p<0.05$) and VSIG2 ($p=0.051$). Plasma protein levels that were particularly higher in VHR participants who had a MACE after 6 months were CCL16 ($p<0.01$), CCL16 ($p<0.01$), IL1RT1 ($p<0.01$), TNFR1 ($p<0.01$), TFF3 ($p<0.01$), ADM ($p<0.01$), CXCL16 ($p<0.01$), TRAILR2 ($p<0.05$), MMP3 ($p<0.05$), LTBR ($p<0.05$), PD-L2 ($p<0.05$), EPHB4 ($p<0.05$), IL2RA ($p<0.05$), SHPS1 ($p<0.05$), IL1RT2 ($p<0.05$), KLK6 ($p<0.05$) and IL4RA ($p<0.05$).

7.3.3 Biomarker associated with MACE occurrence within a year from initial time of admission: Survival analysis

In order to link biomarker levels with MACE development over time, a cox proportional regression model was used and hazard ratios were calculated for all the significant proteins that were selected from the previous step (Table 4). For every unit increase in TNFR1 or IL-6 plasma levels (log levels), the risk of developing MACE increased by 5.47 and 10.29 folds respectively (HR; 5.474; $p<0.05$ and HR: 10.29; $p<0.001$). This significant difference was confirmed by the Proseek assay with an increase of 1.6-fold for TNFR1 and 2.4-fold for IL-6 (HR: 1.603; $p=0.058$ and HR: 2.391; $p<0.0001$). The Proseek assay provides results expressed as normalised protein ratio (NPX).

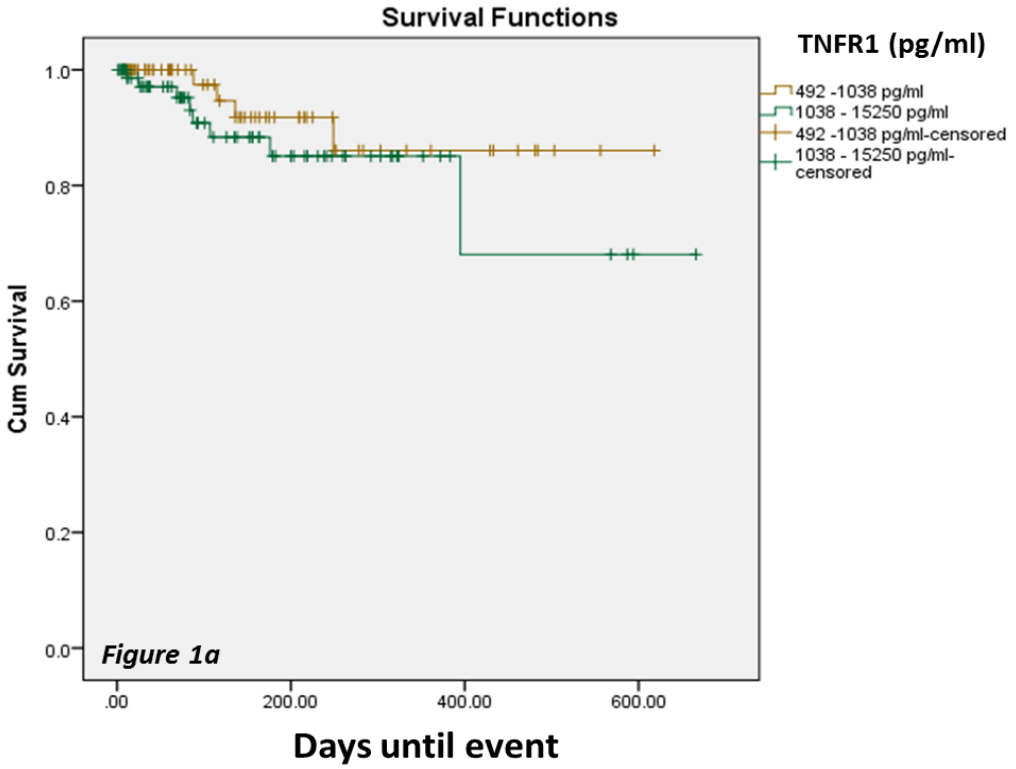
The tested proteins from Olink CVDII and CVDIII panels showed that plasma ST2 (HR: 4.37; $p<0.01$), IL4RA (HR:7.51; $p<0.01$), IL1RT1 (HR:11.89; $p<0.01$), CCL22 (HR: 0.249; $p<0.01$), IL1RT2 (HR: 7.242; $p<0.01$), PDL2 (HR:11.34; $p<0.01$), ADM (HR:3.37; $p<0.01$), THBS2 (HR:13.64; $p<0.01$), CCL16 (HR:5.60; $p<0.05$), RARRES2 (HR:20.30; $p<0.05$), ADAM_TS13 (HR:0.022; $p<0.05$), GDF15 (HR:2.17; $p<0.05$), IDUA (HR:0.324; $p<0.05$), PI3 (HR:1.78; $p<0.05$) and SPON1 (HR:7.96; $p<0.05$) protein levels were linked to MACE occurrence over time (Table 3). In addition, in our present study, CRP plasma levels could not predict MACE risk within a year of admission ($p=0.126$).

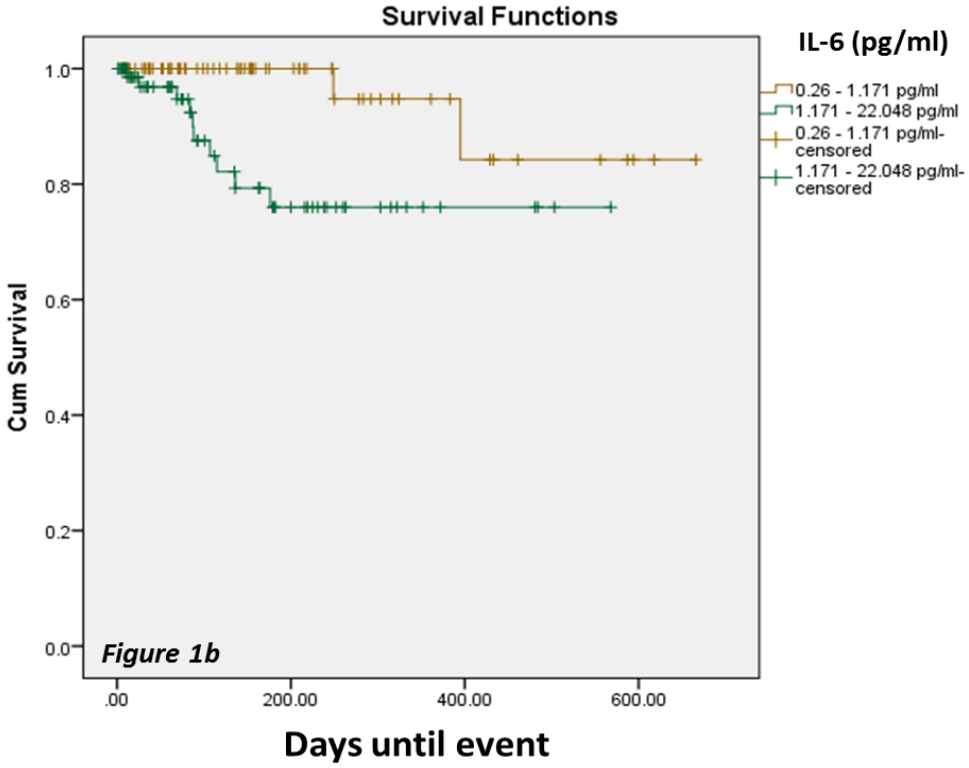
<u>Table 3:</u> Biomarkers hazard ratios and MACE occurrence over time in VHR participants.

Protein	*p value* ***(ranked by p value)***	Hazard ratio	**95.0% confidence interval for hazard ratio**	
			Lower	***Upper***
(Log unit)				
CRP	*0.126*	*1.966*	*0.860*	*4.496*
sTNFR1	*0.045*	5.474	1.042	28.760
IL-6	*0.001*	10.29	2.444	43.334
(NPX unit)				
IL_6	*<0.0001*	2.391	1.469	3.892
ST2	*0.002*	4.367	1.751	10.892
IL_4RA	*0.004*	7.508	1.907	29.558
IL_1RT1	*0.005*	11.884	2.077	67.981
CCL22	*0.007*	0.249	0.091	0.681
IL_1RT2	*0.007*	7.242	1.733	30.262
PD_L2	*0.009*	11.337	1.812	70.944
ADM	*0.01*	3.368	1.334	8.5
THBS2	*0.01*	13.643	1.869	99.608
CCL16	*0.011*	5.605	1.487	21.122
RARRES2	*0.011*	20.302	2.009	205.185
ADAM_TS13	*0.013*	0.022	0.001	0.454
GDF_15	*0.014*	2.175	1.172	4.036
IDUA	*0.034*	0.324	0.115	0.917
PI3	*0.036*	1.776	1.039	3.037
SPON1	*0.046*	7.965	1.035	61.317
REN	*0.054*	1.885	0.988	3.596
TNF_R1	*0.058*	1.603	0.983	2.613
CXCL16	*0.065*	3.309	0.928	11.797
TR	*0.065*	2.163	0.954	4.905
EPHB4	*0.085*	2.882	0.865	9.604
TFF3	*0.089*	1.513	0.939	2.437
TRAIL_R2	*0.101*	1.717	0.899	3.278
PLC	*0.103*	1.827	0.885	3.771
IL2_RA	*0.113*	2.108	0.839	5.296
VSIG2	*0.118*	1.573	0.891	2.777
MMP_3	*0.124*	1.746	0.858	3.554
IGFBP_7	*0.131*	1.900	0.825	4.372
TNF_R2	*0.137*	1.747	0.838	3.643
SHPS_1	*0.138*	2.693	0.727	9.974
LTBR	*0.152*	1.633	0.835	3.192
GRN	*0.154*	3.559	0.622	20.380
IGFBP_2	*0.156*	1.591	0.837	3.021
PAR_1	*0.165*	2.563	0.679	9.667
FGF_21	*0.178*	1.217	0.914	1.619
TNFRSF14	*0.191*	1.456	0.829	2.558
AMBP	*0.192*	5.363	0.431	66.741

IL1RL2	*0.194*	0.383	0.09	1.627
U_PAR	*0.213*	1.87	0.699	5.005
RAGE	*0.217*	2.087	0.650	6.702
KLK6	*0.219*	1.971	0.668	5.822
CCL15	*0.227*	1.598	0.747	3.421
CPA1	*0.245*	1.391	0.797	2.428
AGRP	*0.258*	1.62	0.702	3.736
CD93	*0.403*	1.893	0.424	8.448
CTSD	*0.500*	1.634	0.393	6.803
FGF_23	*0.694*	1.089	0.712	1.666

A cox regression model was used to calculate the hazard ratios for the measured biomarkers and the development of MACE over time. MACE: major adverse cardiovascular events; VHR: very high risk.





<u>Figure 1</u>: Survival analysis (Kaplan Meier curves) exploring the association between TNFR1 and IL-6 plasma protein levels with MACE occurrence over time. *Figure 1a: TNFR1 plasma protein levels were divided according to the median value into two groups. Values between 482 and 1038 pg/ml are represented by the brown line and values between 1038 and 15250 pg/ml are represented by the green line. VHR participants with TNFR1 plasma levels above the median were more likely to develop*

MACE (p<0.05). Figure 1b: IL-6 plasma protein levels were divided according to the median value into two groups. Values between 0.27 and 1.171 pg/ml are represented by the brown line and values between 1.171 and 22.048 pg/ml are represented by the blue line. VHR participants with IL-6 plasma levels above the median were more likely to develop MACE (p<0.01). IL-6: interleukin 6; MACE: major adverse cardiovascular events; TNFR1: tumour necrosis factor alpha receptor 1.

7.4 Discussion

This study highlights the role of potential markers from different inflammatory and immune pathways involved in the initiation and progression of atherosclerosis in MACE prediction. The choice of those biomarkers was based on previous *ex vivo* or *in vivo* studies reporting their association with CVD. Recruited participants were all at very high risk (VHR) which is defined by a 10% risk of cardiovascular death within 10 years (as per the European Society of Cardiology guidelines and the SCORE risk chart) (63).

7.4.1 TNFR1 and IL-6: Potential biomarkers for MACE risk

In this study, TNFR1 plasma protein levels were found to be associated with MACE within one year of admission with particularly higher levels after 6 months of admission. Each one unit increase (log transformed value) in TNFR1 levels was associated with a 5.47-fold increase of MACE (CI [1.042- 28.760]; p<0.05). TNFR1 has been previously associated with death as well as heart failure onset in participants post myocardial infarction (MI) (222). TNFR1 has also been linked to cardiovascular events and mortality in participants with type 2 diabetes (206) as well as in participants with chronic kidney disease (353). The results of this study are in line with previous reports and provide further insight on the value of TNFR1 in MACE prediction.

IL-6 plasma protein levels were associated with MACE with particularly higher levels within 6 months after admission. One unit increase (log transformed value) of IL-6 levels corresponded to a 10.29-fold increase in MACE (CI [2.444-43.334]; p<0.001). It has been

previously shown that IL-6 is a powerful MACE predictor since high plasma level of IL-6 have increased the burden of atherosclerosis and cardiovascular risk in participants without a history of CVD (354). IL-6 was also found to predict MACE and stent thrombosis in participants admitted with unstable angina (355). Since previous studies have associated TNFR1 and IL-6 plasma levels with MACE, these results highlight the need for further studies to evaluate the adoption of such markers in clinical practice for MACE prediction in VHR individuals.

7.4.2 Proteomic approach for MACE prediction

The proteomic approach undertaken in this study revealed a number of potential proteins that were associated with MACE risk in VHR participants with an already established CVD. These proteins included Interleukin 1 receptor-like 1 (IL-1RL1/ST2), Interleukin-4 receptor subunit alpha (IL4RA), Interleukin-1 receptor type 1 (IL1RT1), interleukin 1 receptor 2 (IL1RT2), programmed cell death ligand 2 (PDL2), Adrenomedullin (ADM), Thrombospondin 2 (THBS2), C-C Motif Chemokine Ligand 16 (CCL16), Retinoic acid receptor responder protein 2 (RARRES2), Growth/differentiation factor 15 (GDF15), Elafin (PI3) and spondin 1 (SPON1) which plasma protein levels were higher in VHR participants who developed MACE within a year of admission. In contrast, A disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS13) C-C motif chemokine 22 (CCL22), and alpha-L-iduronidase (IDUA) plasma protein levels were lower in participants who developed MACE within a year of admission compared to those who did not.

IL-1RL1/ST2 is a member of the toll-like receptor superfamily of proteins and is known to be upregulated in myocardial injury to allow an anti-inflammatory response via interleukin 33 (IL-33) in order to preserve cardiac function (356). ST2 has been

suggested to be a marker for acute coronary syndrome (ACS), acute heart failure (357) and cardiac stress and was strongly associated with the development of heart failure post-ACS in the MELRIN-TIMI 36 trial (358). In the present study, every unit increase in ST2 levels was linked to a 4.367-fold increase in MACE risk (HR: 4.367; CI [1.751 – 10.892]; p<0.01). This confirms the previous reports on the role of ST2 in cardiac stress and provides providing further evidence of the potential its role in MACE prediction.

IL-4RA is known to bind to IL-4 and IL-13 which consequently activates macrophages (359) and in the present study, plasma levels were higher in VHR participants who developed MACE where every unit increase in IL-4RA plasma levels was accompanied by 7.508-fold increase in MACE risk (HR: 7.508; CI [1.907 – 29.558]; p<0.01). Little evidence exist on the role of IL-4RA in CAD and MACE, however, polymorphisms in *IL4RA* have been linked to familial hypercholesterolemia (360) which underlines a need to further investigate the role of IL-4RA in CAD and MACE occurrence.

IL-1RT1 (also known as CD121a) is the interleukin receptor for IL-1α and IL-1β and has been suggested to be a novel inflammatory marker for CAD (361) which was also involved in heart remodelling post-MI (333). This highlights the possible role of IL-1RT1 with MACE following an ACS which was shown in the present study (HR: 11.884; CI [2.077 – 6.7981]; p<0.01).

In the present investigation, an association between ADM plasma protein levels and MACE was also highlighted (HR: 3.360; CI [1.334 – 8.5]; p<0.01). This is supported by previous evidence suggesting that ADM is a powerful independent predictor of future cardiovascular events in high-risk participants with a predictive value superior to that of high sensitivity C reactive protein or adiponectin (362). ADM is a vasodilator peptide hormone with hypotensive properties and is known to inhibit cardiac fibroblast

proliferation (363,364). Therefore, the presence of ADM in the plasma might be higher in individuals at risk of MACE in order to counteract the underlying inflammation.

THBS2 in an extracellular matrix protein and its absence has been linked to increased angiogenesis (365) which has been linked to the development of heart failure in participants with CVD (366) and this present study supports the link between THBS2 and MACE risk (HR: 13.643; CI [1.869 – 99.608]; $p<0.01$). Higher levels of THBS2 in VHR individuals could be linked to the development of heart failure.

CCL16 is a chemoattractive cytokine for monocytes and lymphocytes (367) and in this present study, levels were linked to MACE occurrence where every unit increase in CCL16 levels was accompanied by a 5.60-fold increase in MACE risk (CI [1.487 – 21.12]; $p<0.05$). CCL16 has been recently associated with CVD risk in postmenopausal women (368), however, its value in MACE prediction hasn't been assessed yet.

RARRES2, also known as chimerin, is a recently discovered adipokine and has been linked to inflammation and increased oxidative stress in obese participants (369) as well as CVD (370) and acute coronary syndrome (ACS) (371). The results of the present study show that RARRES2 was strongly associated with MACE occurrence with a hazard ratio of 20.302 (CI [2.009 - 205.185]; $p<0.05$). RARRES could be a potential marker for MACE risk in obese individuals.

GDF-15 has immunosuppressive, anti-inflammatory and anti-apoptosis properties and has been linked to CVD, obesity, cancer and kidney injury (372). GDF-15 has been recently suggested as a biomarker for CVD and for MACE (373) with a unique capacity in capturing CVD development, progression, and prognosis, which is not the case for clinical risk predictors and other biomarkers (374). This finding is consolidated in the present study (HR: 2.175; CI [1.1172 – 4.036]; $p<0.05$). GDF15 is shown to be

upregulated in many disease processes, nevertheless, it is unclear whether it leads to the further progression of the disease or provides protection against the disease (375).

PI3 is an endogenous inhibitor of neutrophil-derived elastases which has been shown to present anti-inflammatory activity in pre-clinical models of inflammatory vascular injury (376) and in this present study, PI3 was able to predict MACE risk (HR: 1.776; CI [1.039 – 3.037]; $p<0.05$). In CVD, higher levels of anti-inflammatory mediators, such as Pl3, will attempt to counteract pro-inflammatory mediators and reflect a self defence mechanism of the body against chronic inflammation.

On the other hand, there is little evidence of the role of IL-1RT2, PD-L2 and SPON1 in CAD and MACE prediction. Nonetheless, the current study showed that IL-1RT2, PD-L2 and SPON1 were linked to an increased MACE risk in VHR participants (HR: 7.242; CI [1.733 – 30.262]; $p<0.01$, HR: 11.337; CI [1.812 – 70.944 $p<0.01$ and HR: 7.965; CI [1.035 – 61.37]; $p<0.05$ respectively). IL-1RT2 binds IL-1α and IL-1β (377) whereas PD-L2 is known to downregulate the immune system and promoting self-tolerance (378) and SPON1 has been shown to be involved in smooth muscle cell growth and angiogenesis (379). The role of such proteins should be further explored in CVD.

ADAMTS13 cleaves von Willebrand factor in order to decrease its activity and inhibits coagulation (380). It is established that low levels of ADAMTS13 levels contribute to haematological and cardiovascular disorders (312) and a severe deficiency in ADAMTS13 results in the clotting disorder known as thrombotic thrombocytopenic purpura (381). Our results are in line with these previous reports and show that a unit increase in ADAMTS13 levels was associated with a 97.8 % reduction in MACE risk (HR: 0.022; CI [0.001 – 0.454]; $p<0.05$). High level of ADAMTS13 in VHR participants at risk of MACE might be associated with an attempt to counteract a pro-thrombotic state.

Furthermore, each unit increase in CCL22 and IDUA levels was associated with a 75.1% and a 67.7% reduction in MACE risk (HR: 0.249; CI [0.091 – 0.681]; p<0.01 and HR: 0.324; CI [0.115 – 0.917]; p<0.05 respectively). CCL22, mainly produced by macrophages, triggers the migration of regulatory T cells and is involved in infections and cancer (382). IDUA, on the other hand, is a lysosomal enzyme and, when defective, can cause a lysosomal storage disease known as Hurler or Scheie syndrome (383). There is little evidence of the role of CCL22 and IDUA in CVD and this requires further exploration as it appears that lower levels of those proteins are associated with MACE.

In summary, combining a set of proteins that showed a significant association with MACE risk would allow an early detection to optimise treatment of vulnerable patients. The value of such a panel is reflected by a logistic regression model combining all the previously significant proteins (n=15) which was able to significantly predict the occurrence of a follow-up MACE in 94.2% of the cases (p<0.0001). This highlights the importance of a multimarker approach in MACE prediction where a panel combining multiple proteins can overcome any individual limitations of clinically relevant proteins in terms of low specificity and sensitivity (384).

7.4.3 Strengths and Limitations

Refer to **section 3.4.3 in Chapter 3**.

In terms of potential biases resulting from the biochemical measurements used in the present study, it is important to consider that IL-13, IL-1β, IL-4 and natriuretic peptides (BNP and pro-NT BNP) plasma levels were below the limit of detection of the assays used in this study and therefore they were excluded from the analysis. There were 4 values

that were below the limit of detection for INF-γ assay, 2 for the IL-10 assay, 21 for the IL-2 and 9 for the IL-12p70 assay which also had a high coefficient of variation.

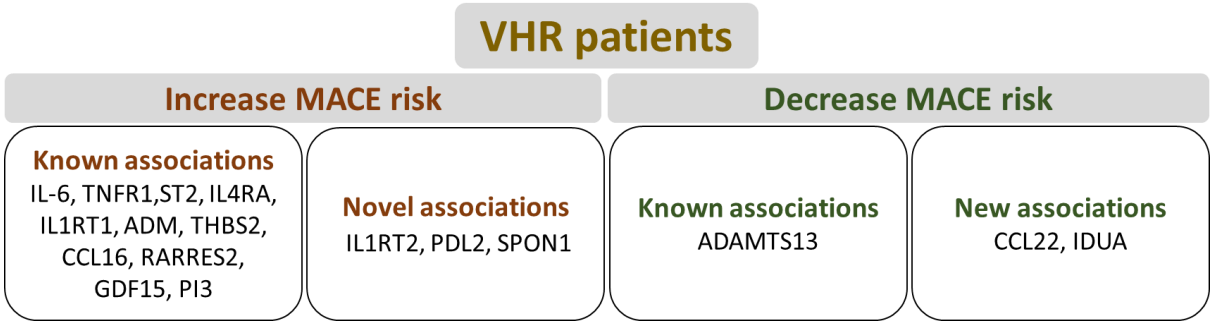


***Figure 2:* Previously established and novel associations between inflammatory proteins and MACE risk.** *MACE: major adverse cardiovascular events; VHR: very high risk.*

7.5 Conclusion

In conclusion, the present investigation highlights previously reported and novel associations between several inflammatory proteins and MACE risk. The candidate proteins explored in the study should be explored further in large prospective studies to assess their clinical value and possibly included in a biomarker panel to assess MACE risk. If adopted in clinical practice, such biomarkers can reduce the number of CVD participants who develop recurrent events by providing them with a better medical and clinical management.

Chapter 8

Exploring Analytical and Technical Parameters during Biomarker Development

Abstract

Background: Despite many biomarkers being associated with cardiovascular disease (CVD), only few are adopted into clinical practice. Markers that show to effectively predict cardiovascular risk in clinical studies often face many challenges when it comes to their implementation into a clinical practice. Some of these challenges are due to a lack of specificity and sensitivity of the tested biomarkers. In addition, changes in biomarker levels according to sampling site and storage conditions are crucial to consider in biomarker development. Therefore, we undertook the present investigation to evaluate some of the common characteristics that should be present in an ideal biomarker.

Methods: Participants were recruited from the cardiac catheterisation laboratory or by email advertisement. Arterial and venous blood samples were collected and were measured in different experimental conditions. Blood samples from different sampling sites were also collected in a number of participants. All participants were assigned a risk score using the European Society of Cardiology (ESC) SCORE risk chart. Group 1 was defined as very high risk participants (VHR) with a 10-year risk SCORE ≥10% risk of fatal cardiovascular disease (CVD). Group 2 were defined as low, moderate and high risk participants (non-VHR) with a 10-year risk SCORE <10% risk of fatal CVD. TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels were measured by ELISA. INF-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNFα plasma protein levels were measured using MSD® MULTI-SPOT Assay System. *TACE* mRNA levels were measured by quantitative real-time PCR. CVDII and CVDIII panels were measured in the plasma by multiplex proximity extension assays (PEA) by Olink® proteomics.

Results: TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels were not significantly different between blood samples taken from the peripheral artery and the peripheral

vein (p=0.553; p=0.404; p=0.358 and p=0.792 respectively). However, TNFR1 and TNFR2 plasma protein levels were higher in the samples taken from the peripheral artery compared to the ones that were taken from the central vein and the coronary sinus ($p<0.05$ and $p<0.01$) and were also higher in samples taken from the central vein compared to the coronary sinus ($p<0.01$ and $p<0.01$). TACE plasma levels were not different when compared between serum samples (p=0.289), samples that were subject to different centrifugation speeds (p=0.403), samples measured on the day of collection and after 10 days or 78 days (p=0.373) and samples measured on two occasions (p=0.537). TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels were not statistically different when measured over time in participants before and after a revascularisation intervention. In terms of specificity and sensitivity, the proteins that significantly distinguished between VHR and non-VHR participants with a high sensitivity and specificity were Growth/differentiation factor 15 (GDF-15) (AUC:0.883; $p<0.0001$), TNF related apoptosis-inducing ligand receptor 2 (TRAIL-R2) (AUC:0.869; $p<0.0001$), Matrix metalloproteinase-7 (MMP7) (AUC:0.865; $p<0.0001$), TNFα (AUC:0.835; $p<0.0001$), Matrix metalloproteinase-12 (MMP12) (AUC:0.825; $p<0.0001$), Urokinase receptor (UPAR) (AUC: 0.814; $p<0.0001$), IL-6 (AUC: 0.813; $p<0.0001$), Renin (REN) (AUC: 0.809; $p<0.0001$) and IL-8 (AUC: 0.804; $p<0.0001$).

Conclusion: In conclusion, this study highlights the importance of evaluating different experimental conditions and measurement methods during biomarker development. This process should take place in parallel with assessing the clinical value of any potential marker. These crucial steps could help uncover any experimental or technical problems that normally challenge the translation of any new biomarker into clinical practice.

8.1 Introduction

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention; a definition that was standardised in 2001 by the National Institutes of Health (NIH) working group. A biomarker can be a gene mutation, a polymorphism, a protein, or other molecule or clinical measurement that indicates a given disease state. Therefore, biomarkers may be measured in a biosample (such as a blood, urine, or tissue test), recorded or obtained from a person (blood pressure, ECG, or Holter monitor) or from an imaging test (echocardiogram or CT scan) (112). Biomarker research has received a lot of attention over the past couple of decades. However, biomarkers that have shown efficiency in clinical trials are rarely adopted into the routine clinical practice. This is because the translation of biomarker research to clinical application faces many challenges in terms of the interpretation of biomarker levels, standardisation of measurement methods, optimisation of regulations and there are many difficulties overcoming ethical issues (283).

Non-specificity of biomarkers is one of the many problems that exists in biomarker research in cardiovascular disease (CVD) (385). In addition, the prognostic value of various already adopted biomarkers (such as troponins and C reactive protein) is not clear since these proteins are not usually measured as part of a follow up strategy in patients with an established CVD (145,386). In biomarker development, many issues need to be addressed beside the clinical relevance of a certain biomarker. In addition to accuracy, precision, high sensitivity and specificity, the biomarker needs to be acceptable to the patient, tested in a large number of individuals, internationally standardised and it needs to add value to the clinical assessment (113,137).

Furthermore, parameters such as changes in biomarker levels according to sampling sites, over time and in different storage conditions are crucial.

Tumour necrosis factor alpha converting enzyme (TACE) is a membrane bound protein responsible for the cleavage of tumour necrosis factor alpha (TNFα) and both of its receptors tumour necrosis factor alpha receptor 1 (TNFR1) and 2 (TNFR2). Several clinical studies have suggested the possible use of such biomarkers to improve the current tools used in CVD risk assessment (170,171,206,207). TACE endogenous inhibitor metalloproteinase inhibitor 3 (TIMP3) has been previously shown to be downregulated in circulating human monocytes in people at high risk of diabetes and atherosclerosis (221). In addition, many inflammatory markers such as interleukin 6 (IL-6) (240), interleukin 8 (IL-8) (246) and interferon gamma (INF-γ) (248), known to play a role in atherosclerosis initiation and progression, have been measured in CVD patients. Nevertheless, there are no studies that have evaluated the practicalities in terms of sensitivity, specificity, accuracy and measurement variability of using such biomarkers in clinical practice.

In this present study, the explored biomarkers have been measured over time, in different sampling sites and in different storage conditions. The aim was to assess several technical and experimental issues related to biomarker development.

8.2 Participants and Methods

Refer to **section 2.1** in **Chapter 2** for a detailed description of the participant recruitment process, the risk score used and the methods for blood processing.

8.2.1 Demographic information

Refer to **section 3.2.1** in **Chapter 3** for a detailed description of the demographic information that was collected in this study.

8.2.2 Participants blood sample collection

Blood (35 ml) was collected from all recruited participants in a fasting state. Three 8 ml EDTA tubes for the research lab (VACUETTE K3E K3EDTA – 455036), one 4 ml serum tube for the research lab (VACUETTE Z serum clot activator - 454092), one 4 ml EDTA tube for hospital lab (to measure the full blood count (FBC)) (VACUETTE K3E K3 EDTA – 454036) and one 3.5 ml of serum separator tube (yellow cap) (to measure CRP, renal function and cholesterol levels) (BD Vacutainer SST^{TM} II advance – 367956) were collected. For the participants recruited from the catheterisation laboratory in Altnagelvin Hospital, blood was collected from the sheath inserted into the radial artery (or, on occasions, the femoral artery when the radial artery couldn't bet accessed). The arterial blood was collected straight into a 50-ml syringe and immediately transferred into the blood tubes using a 21 G syringe (BD microlane 3 – 21G 1 ½ Nr.2 0.8 x 40 mm – 304432). Blood samples were immediately cooled and centrifuged and the plasma was aliquoted and frozen at −80◦C until further analysis (Figure 1).

From a subset of participants recruited from the catheterisation laboratory in Altnagelvin Hospital, a total of 40 ml of venous (peripheral vein) (V=30 ml) and arterial (peripheral artery) blood (V=10 ml) was collected. This was to compare the effect of the sampling site on the studied markers. The venous blood was collected by a trained phlebotomist prior to the angiogram procedure using the same blood collection material mentioned above (Figure 1).

From another subset of participants recruited from the catheterisation laboratory in Altnagelvin Hospital, 10 ml of blood was collected from different sampling sites by the consultant cardiologist. The different sampling sites include the peripheral artery (PA) (radial or femoral artery), central vein (CV) (femoral vein), central coronary artery (CCA), culprit lesion (when present), coronary sinus, a post-balloon sample, a post-PCI sample and a post-heparin sample (Figure 1).

Participants recruited among the hospital and Ulster University staff were received at the clinical translational research and innovation centre (C-TRIC) and a 35 ml of venous blood was collected from the peripheral vein by a trained phlebotomist using a butterfly needle (VACUETTE safety blood collection set 21G x 3/4 '' 0.9 x 19 mm – 450091) as well as a luer adapter (Greiner bio one luer adapter – 20G 16C05A) and a BD Vacutainer (Figure 1).

A venous (peripheral vein) blood sample was collected from some of the previously recruited participants who volunteered to give a second blood sample after one year of initial blood sample collection. A volume of 8 ml of venous blood was collected by a trained phlebotomist using the same material mentioned above (Figure 1).

8.2.3 Measurement of soluble TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels

Refer to **section 2.6.1, 2.7.1, 2.8.1, 2.9.1 in Chapter 2.**

8.2.4 Measurement of TNFα, IFN-γ, IL-2, IL-6, IL-8, IL-10 and IL-12p70 plasma protein levels

Refer to **section 2.10 in Chapter 2.**

8.2.5 Method for analysing samples using the Proximity Extension Assay (PEA) technology - Proseek® Multiplex provided by O-Link Proteomics (CVDII and CVDIII)

Refer to **section 2.14 in Chapter 2.**

A detailed definition of the covariates is given in **section 3.2.7 in Chapter 3**

8.2.6 Statistical methods

Data analysis was performed using GraphPad Prism 6 software© and IBM® SPSS version 23 software (SPSS Inc., Chicago, IL). Variables that did not follow normal distribution were transformed using a logarithmic function to allow their use in parametric methods of analysis. Continuous variables were expressed as mean ± SD whereas categorical variables were expressed as percentages. Unpaired and paired student tests were conducted according to the type of analysis. Receiver operating characteristics (ROC) curves were calculated for the significant proteins in order to assess the sensitivity and specificity of the test. Statistical significance was defined as values of $p<0.05$ (two-tailed).

8.3 Results and Discussion

8.3.1 Participant recruitment and blood sample collection

Refer to section **3.3.1 in Chapter 3** and **Table 1 and 2 in Chapter 3**.

Among the participants recruited from the catheterisation laboratory in Altnagelvin Hospital, arterial (n=153) or venous (n=47) blood was collected from Group 1. Arterial and venous blood (n=38) were collected from Group 2. Blood (n=6) was collected from different sampling sites (which included the peripheral artery (PA) (radial or femoral artery), central vein (CV) (femoral vein), central coronary artery (CCA), culprit lesion (when present), coronary sinus, a post-angioplasty sample, a post-PCI sample and post-heparin sample (from the peripheral artery) from Group 3. Among the participants recruited from the hospital and Ulster University staff, venous blood (n=100) was collected from all individuals (Group 4). A follow-up blood sample (n=16) was collected from participants who were initially recruited from the catheterisation lab in Altnagelvin Hospital and among the hospital and Ulster University staff (Group 5) (Figure 1).

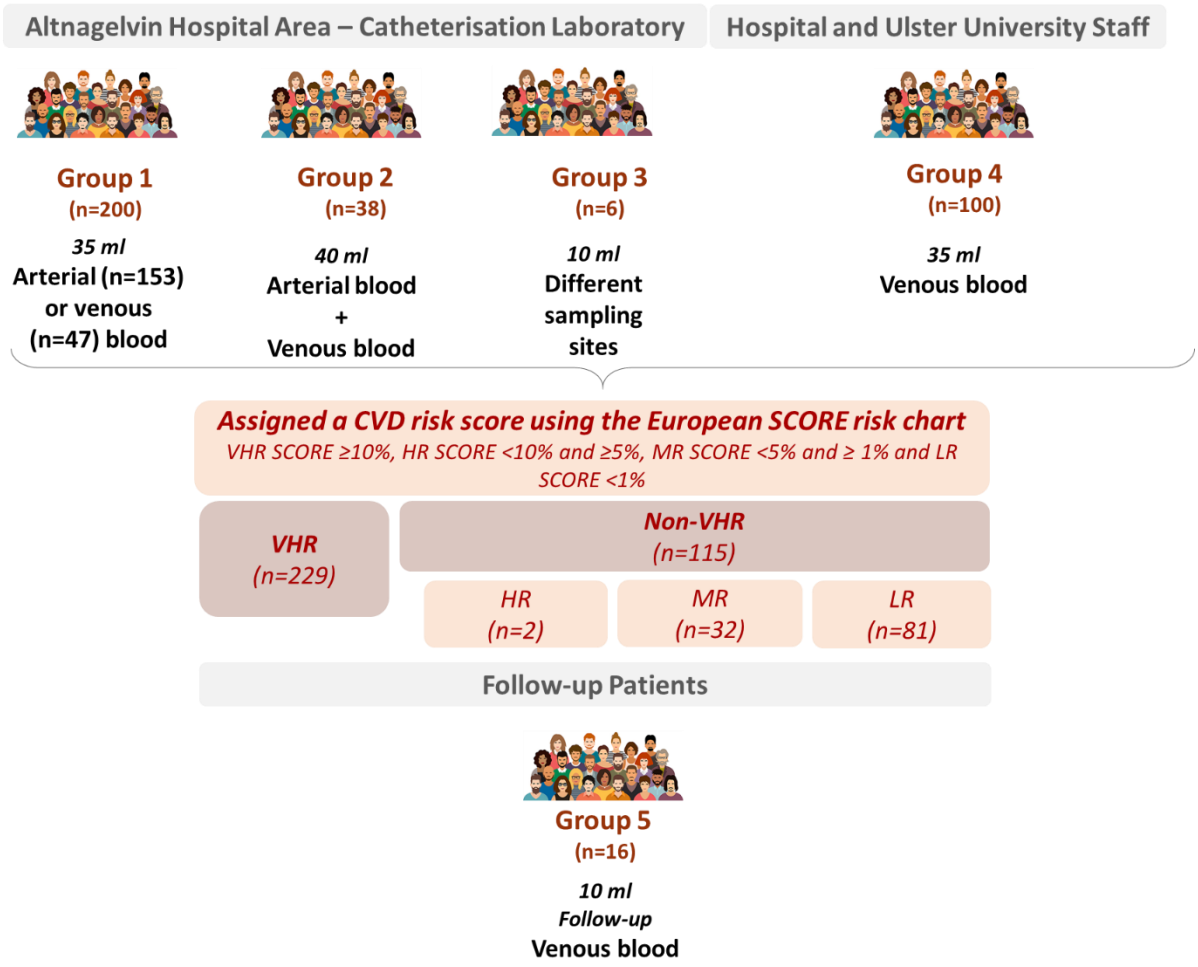


***Figure 1*: Types and volumes of the blood samples collected from participants recruited from the catheterisation laboratory in Altnagelvin Area Hospital and from the hospital and Ulster University staff.** *CVD: Cardiovascular disease, HR: high risk, LR: low risk, MR: moderate risk, SCORE: Systematic COronary Risk Evaluation; risk, VHR: very high*

<u>Table 1:</u> TACE, TNFR1, TNFR2 and TIMP3 protein levels measured in different types of samples

Type of sample	TACE	TNFR1	TNFR2	TIMP3
Arterial blood	✓	✓	✓	✓
Venous blood	✓	✓	✓	✓
Arterial and venous blood from same participant	✓	✓	✓	✓
Different sampling sites from same participant	✓	✓	✓	✓
Serum	✓			
Timepoint 1	✓	✓	✓	✓
Timepoint 2	✓	✓	✓	✓
Spun down plasma	✓			
Fresh plasma measured on the day of collection	✓			

TACE protein levels were measured in arterial samples, venous samples, arterial and venous samples from the same participants, in different sampling sites, in the serum, on timepoint one and 2, in plasma samples after being spun down, and in plasma samples collected on the day. TNFR1, TNFR2 and TIMP3 protein levels were measured in arterial samples, venous samples, arterial and venous samples from the same participants, in different sampling sites, on timepoint one and 2. TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: Metalloproteinase inhibitor 3; TNFα: Tumour necrosis factor alpha; TNFR1: Tumour necrosis factor alpha receptor 1; TNFR2: Tumour necrosis factor alpha receptor 2.

<u>Table 2:</u> Assay precision and coefficient of variation for the measured biomarkers.

Assay precision	**Inter-assay CV (%)**	**Intra-assay CV (%)**
TACE ELISA (RAB0003 SIGMA)	19.68	11.91
TIMP3 ELISA (abcam ab119608)	26.57	5.49
TNFR1 ELISA (R&D - DRT 100)	8.62	4.20
TNFR2 ELISA (R&D - DRT200)	5.07	4.20
MSD® MULTI-SPOT Assay System - K15049D		
INF-γ	16.30	5.90
IL-10	13.97	6.93
IL-12p70	24.06	23.68
IL-2	40.16	20.98
IL-6	8.27	4.61
IL-8	16.11	3.37
TNFα	26.51	3.16

CV: coefficient of variation; IL-10: Interleukin 10; IL-12p70: Interleukin 12 hetoremer 70; IL-2: Interleukin 2; IL-6: Interleukin 6; IL-8: Interleukin 8; INF-γ: Interferon gamma; TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: Metalloproteinase inhibitor 3; TNFα: Tumour necrosis factor alpha; TNFR1: Tumour necrosis factor alpha receptor 1; TNFR2: Tumour necrosis factor alpha receptor 2.

8.3.2 Biomarker levels were not statistically different between samples taken from the peripheral artery vs. the peripheral vein

To explore whether TACE, TNFR1, TNFR2 and TIMP3 protein levels were subject to differences between sampling sites, blood samples taken from the peripheral artery were compared to blood samples taken from the peripheral vein. While the peripheral vein might be more relevant in routine practice, in cardiology, the peripheral artery could be also useful when it comes to cytokine levels. This is because arterial blood provides the circulating levels of cytokines prior to the removal by the body tissues (387).

Results showed that there was no significant difference between TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels when compared between blood samples taken from the peripheral artery and the peripheral vein ($p=0.553$; $p=0.404$; $p=0.358$ and $p=0.792$ respectively) (Table 3). TACE plasma levels were previously measured in ANCA vasculitis and Alzheimer's disease (176,177), TIMP3 plasma levels were measured in patients with oral cancer (388) and TNFR1 and TNFR2 plasma levels were measured in patients with CVD. However, the value of such markers as potential biomarkers in CVD hasn't been assessed yet as the authors did not evaluate the sensitivity and specificity of their assay and did not explore whether the sampling site affects the measurement. Since atherosclerosis mainly affects the body's arteries, evaluating biomarker levels in different sampling sites is crucial as some markers tend to be higher in the arterial compared to the venous circulation (389).

***Table 3:* Biomarker levels in blood samples taken from the peripheral artery and the peripheral vein.**

Plasma protein levels (pg/ml)	*Number of samples*	Arterial *(pg/ml)*		Venous *(pg/ml)*		*p value*
		Mean	Std. deviation	Mean	Std. deviation	
TACE	38	377.3	874.2	483.7	1061	0.462
TNFR1	18	1214	607.1	1383	591.5	0.405
TNFR2	18	2795	1355	3344	2101	0.358
TIMP3	20	2668	2638	2859	1835	0.792

An unpaired Student t-test was performed. TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: Metalloproteinase inhibitor 3; TNFR1: Tumour necrosis factor alpha receptor 1; TNFR2: Tumour necrosis factor alpha receptor 2.

8.3.3 TNFR1 and TNFR2 plasma levels were statistically different according to sampling sites

The present study is the first to compare plasma TNFR1 and TNFR2 protein levels between the peripheral artery, the central vein and the coronary sinus. This was done in order to investigate whether the concentrations of some markers vary within the blood circulation. In addition, differences in protein levels were explored before and after angioplasty and PCI procedure and as a consequence of heparin administration. Results demonstrated that TNFR1 and TNFR2 plasma protein levels were higher in the samples that were taken from the peripheral artery compared to the ones that were taken from the central vein and the coronary sinus (1088 ± 213.6 pg/ml vs. 1047 ± 198.5 pg/ml and 952.8 ± 189.7 pg/ml; $p<0.05$ for TNFR1 and 2621 ± 980.6 pg/ml vs. 2300 ± 783.4 pg/ml and 1967 ± 721.3 pg/ml; $p<0.01$ for TNFR2) (Figure 2). Furthermore, TNFR1 and TNFR2 plasma levels were higher in the central vein compared to the coronary sinus (1047 ± 198.5 pg/ml vs. 952.8 ± 189.7 pg/ml; $p<0.01$ and 2300 ± 783.4 pg/ml vs. 1967 ± 721.3 pg/ml; $p<0.01$) (Figure 2 and Table 4). It was previously shown that the expression of TNFR1 in the arterial wall promotes atherosclerosis (390). Also, cerebral ischemia is known to induce the expression of TNFα and its receptors in the walls of cerebral arteries (391). This might result in a higher proportion of soluble TNFR1 and TNFR2

proteins that are shed in the peripheral artery compared to the central vein and coronary sinus and the processes behind this need further understanding. However, it is suggested that arterial blood reflects the circulating levels of cytokines prior to the removal by the body tissues (387) which would indicate that some markers tend to be higher in the arterial circulation compared to the venous circulation. On the other hand, it appears that post angioplasty, and post PCI, TNFR1 plasma levels seem to increase whereas TNFR2 plasma levels decreased. However, the difference did not reach statistical significance probably due to the low number of participants tested (Table 6).

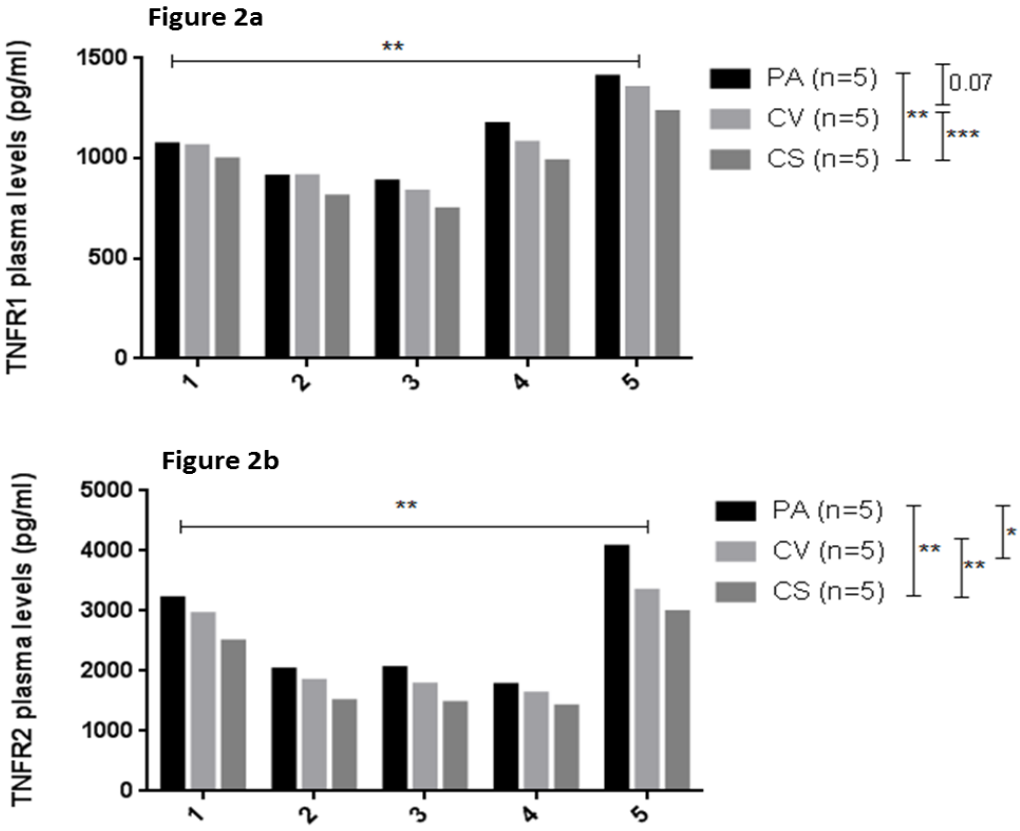


***Figure 2:* TNFR1 and TNFR2 plasma levels are higher is samples taken from the peripheral artery (PA) compared to samples taken from the central vein (CV) and the coronary sinus (CS).** *A paired Student t-test was performed. TNFR1 and TNFR2 plasma levels were measured by ELISA in the different sampling sites taken from individuals at very high risk of cardiovascular risk. TNFR1: tumour necrosis factor alpha receptor 1; TNFR2: tumour necrosis factor alpha receptor 2.*

***Table 6*: TACE, TNFR1, TNFR2 and TIMP3 plasma protein levels across different sampling sites in VHR participants.**

Protein levels (pg/ml) in different sampling sites	**Peripheral artery (n=6)**		**Central vein (n=5)**		**Coronary sinus (n=5)**		***p value***	**Culprit lesion (n=3)**		**Culprit lesion post angioplasty (n=3)**		**Culprit lesion post PCI (n=3)**		***p value***
	Mean	*Std. deviation*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*ANOVA (paired)*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*ANOVA (paired)*
TACE	1773	3145	1809	3260	1652	3010	0.3417	173.7	200.9	150.1	190.9	131.3	227.5	0.4274
TNFR1	**1088**	213.6	1047	198.5	952.8	189.7	0.0019	753.9	58.28	785.7	187.1	796	118.4	0.7171
TNFR2	**2621**	980.6	2300	783.4	1967	721.3	0.0056	1402	150	1387	36.36	1375	119.9	0.8529
TIMP3	2557	1703	2319	1314	2946	859.9	0.3523	5476	2101	3492	1753	4289	787.1	0.4299
	Peripheral artery (n=3)		**Central vein (n=3)**		**Coronary sinus (n=3)**		**Culprit lesion (n=3)**		**Culprit lesion post angioplasty (n=3)**		**Culprit lesion post PCI (n=3)**		***p value***	
	Mean	*Std. deviation*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*Mean*	*Std. deviation*	*ANOVA (paired)*	
TACE	203.9	230.2	183.6	253.3	127.9	221.6	173.7	200.9	150.1	190.9	131.3	227.5	0.412	
TNFR1	987.7	158.3	940.5	124.4	846.2	123.8	753.9	58.28	785.7	187.1	796	118.4	0.0906	
TNFR2	**1945**	157.7	1739	110.8	1456	46.52	1402	150	1387	36.36	1375	119.9	0.0173	
TIMP3	3403	1748	3030	1220	3300	919.8	5476	2101	3492	1753	4289	787.1	0.3226	

Highlighted p values are significant. Values in bold are the highest among the compared cells. PCI: percutaneous coronary intervention, TACE: tumour necrosis factor alpha converting enzyme; TIMP3: metalloproteinase inhibitor 3inhibitor; TNFR1: tumour necrosis factor alpha receptor 1; TNFR2: tumour necrosis factor alpha receptor 2; VHR: very high risk.

8.3.4 TACE levels were not statistically different in samples measured in different experimental conditions

In order to determine the effect of different experimental conditions on TACE levels, TACE protein levels were measured in plasma and serum as well as before and after a centrifugation step and over time. Our data demonstrated that there was no significant difference in TACE protein levels that were measured in the plasma compared to the serum ($p=0.289$). Moreover, centrifuging the plasma sample for 5 min at 15,000 RCF, in order to pellet an microparticles (392), did not affect TACE detection ($p=0.403$). Furthermore, there was also no significant difference when measuring TACE plasma protein levels in a sample on the day of collection, after 10 days or after 78 days of collection ($p=0.373$). In addition, measuring the same plasma sample on two occasions did not have a significant difference in terms of protein levels ($p=0.537$) (Table 5). These results show that TACE plasma protein levels are not subject to experimental and technical changes. These characteristics are important when it comes to biomarker development since an ideal biomarker needs to be subject to little experimental variability and be stable in different measurement conditions (137) (Table 5).

***Table 5*: TACE protein levels measured in different experimental conditions**

Experimental conditions	TACE plasma levels (pg/ml)			*p value*
	Number of samples	Mean	Std. deviation	*Student paired t test*
Plasma	13	497.1	865.7	0.2894
Serum		366.9	759.9	
Before centrifuging plasma	3	1018	1710	0.4034
After centrifuging plasma		886.3	1535	
Fresh samples	6	1803	3116	0.3729
Measured after 10 days		714.6	877.9	
Measured after 78 days		873.4	1066	
Measurement 1	34	1110	2237	0.5371
Measurement 2		901.3	1545	
Days after measurement 1 after sample collection (average in days)		40		
Days after measurement 2 after sample collection (average in days)		302		
				Pearson's correlation analysis
Difference between measurement 1 and 2		381.9795588	1148.606872	0.136
Days between measurement 2 and measurement 1 (average in days)		364		

Samples were centrifuged for 15000 RCF for 5 min. TACE: Tumour necrosis factor alpha converting enzyme

8.3.5 Biomarker levels in follow up participants

To investigate whether protein levels change over time in the recruited participants, a total of 13 VHR participants volunteered to provide a second blood sample after one year of recruitment. Among these participants, 6 had underwent a PCI or coronary artery bypass grafting (CABG) between the timepoint of the first and the second blood sample. None of the measured biomarkers had significantly different plasma protein levels when compared between the first and the second sample (Table 6). Although inflammatory markers have been shown to increase after a PCI or a CABG (393–395),

the duration of this increase remains unclear. In most of the studies, the increase in inflammatory markers have been measured directly after the PCI or the CABG procedure and up to two 2 days after the procedure and haven't been measured over time. In addition, a PCI or a CABG procedure are normally accompanied by the initiation of a drug therapy and therefore, it is quite challenging to assess the efficacy of a PCI or a CABG therapy alone without the biased beneficial effect of the drug therapy. Furthermore, the number of the follow-up participants that volunteered for a blood sample following a PCI or a CABG procedure in this present study was rather low and therefore, a statistical difference was not observed. However, follow-up participants are still being recruited in this study.

***Table 6*: Biomarker levels in a follow up blood sample from VHR participants after PCI or CABG**

Plasma levels (pg/ml)	**Before PCI/CABG** *(n=6)*		**After PCI/CABG** *(n=6)*		***p value***
	Mean	*Std. deviation*	*Mean*	*Std. deviation*	
TACE	2358.25	3633.88	2583.90	3621.83	0.215
TNFα	3.72	1.22	2.78	0.53	0.063
TNFR1	974.00	234.00	1102.85	177.34	0.237
TNFR2	2438.50	693.98	2597.33	774.22	0.653
TIMP3	2473.67	1536.62	1100.67	496.59	0.109
INF-γ	5.91	5.60	7.08	5.48	0.419
IL-10	0.22	0.15	0.52	0.56	0.288
IL-12p70	0.36	0.29	0.20	0.09	0.172
IL-2	0.21	0.09	0.23	0.08	0.504
IL-6	1.43	1.26	1.22	0.61	0.651
IL-8	8.01	8.86	6.09	2.01	0.586

An unpaired student t-test was performed. CABG: coronary artery bypass grafting; IL-10: Interleukin 10; IL-12p70: Interleukin 12 hetoremer 70; IL-2: Interleukin 2; IL-6: Interleukin 6; IL-8: Interleukin 8; INF-γ: Interferon gamma; PCI: percutaneous intervention; TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: Metalloproteinase inhibitor 3; TNFα: Tumour necrosis factor alpha; TNFR1: Tumour necrosis factor alpha receptor 1; TNFR2: Tumour necrosis factor alpha receptor 2.

8.3.6 ROC curves to assess sensitivity and specificity of the measured biomarkers in differentiating between VHR and non-VHR individuals

In addition to the previously measured proteins, a proteomic approach was used to identify biomarkers that were able to differentiate between VHR and non-VHR participants. Refer to **Table 8** in **section III** of the appendix for the full list of proteins, area under the curve (AUC) and p values. To test the sensitivity and specificity of the best candidate proteins, receiver operating characteristic (ROC) curves were generated and the AUC was calculated (Table 7). Growth/differentiation factor 15 (GDF-15), also known as macrophage inhibitory cytokine-1, has a major role in regulating inflammation and apoptosis in injured tissues (375). GDF-15 has been proposed as a new biomarker for CVD, for stable coronary artery disease (CAD), acute coronary syndrome (ACS) and heart failure (308). However, the evaluation of its possible role as a biomarker hasn't been previously assessed. In this present study and among all the measure proteins, GDF-15 has shown to have the highest sensitivity and specificity in differentiating between VHR and non-VHR individuals (AUC:0.883; $p<0.0001$) suggesting a promising role in biomarker development. In addition, TNF related apoptosis-inducing ligand receptor 2 (TRAIL-R2) is suggested to cause apoptosis (396), however its role in CVD risk assessment needs further exploration (AUC:0.869; $p<0.0001$). Matrix metalloproteinase-7 (MMP7) is known to contribute to plaque instability in atherosclerosis (297) whereas Matrix metalloproteinase-12 (MMP12) has been shown to be a candidate molecule for the prevention and treatment of cardiometabolic diseases (302). These two metalloproteinases which are involved in the breakdown of the extracellular matrix and could be promising for biomarker development since they demonstrated a high specificity and sensitivity in identifying cardiovascular risk

(AUC:0.865; p<0.0001 and AUC:0.825; p<0.0001, respectively). Urokinase receptor (UPAR) (AUC: 0.814; p<0.0001) has been suggested as a biomarker for CVD in patients with chronic kidney disease (CKD) (349) whereas Renin (REN) (AUC: 0.809; p<0.0001) plasma activity was recently linked to increased risk of major adverse cardiovascular events (MACE) and congestive heart failure in patients with high systolic blood pressure (307). In addition, TNFα (AUC:0.835; p<0.0001), IL-6 (AUC: 0.813; p<0.0001), and IL-8 (AUC: 0.804; p<0.0001) demonstrated high sensitivity and specificity in differentiating between VHR and non-VHR individuals. All the previously mentioned proteins had an AUC > 0.8. In is noteworthy that CRP levels had and AUC of 0.667 (CI: 0.607 – 0.729; p<0.0001) which was lower compared to the aforementioned proteins. Such proteins could be potentially added to current CVD diagnostic tools in order to improve current CVD risk assessment.

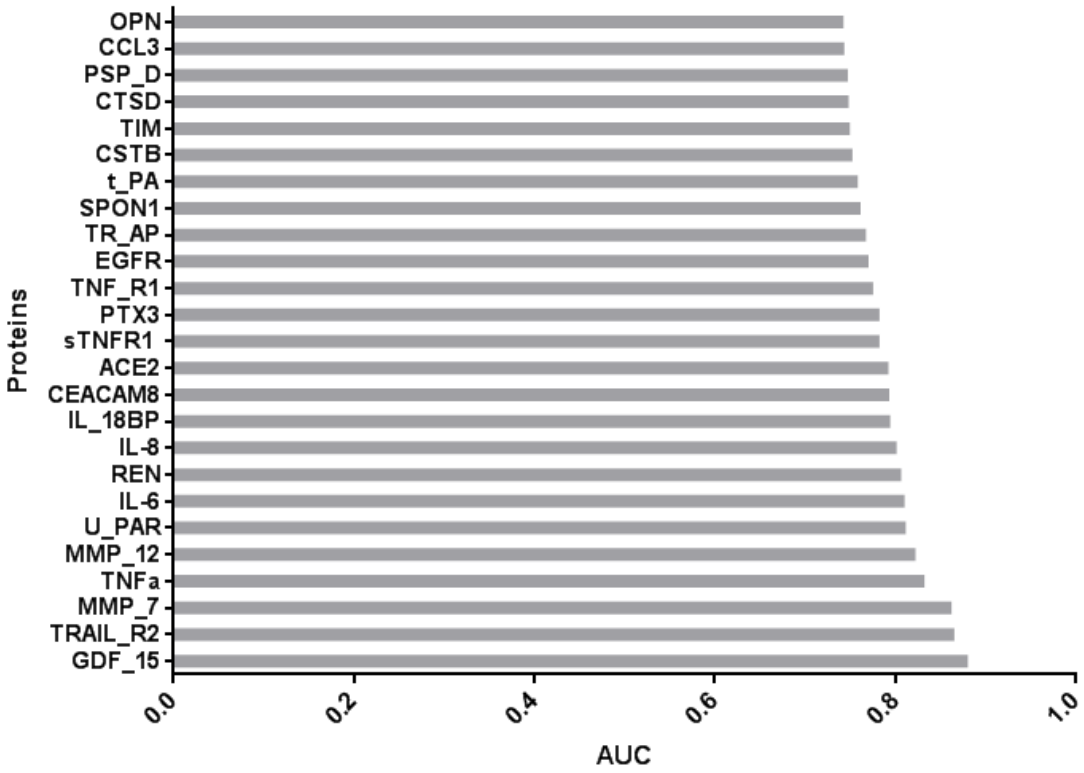


***Figure 3:* AUC values of the proteins that significantly differentiated between VHR and non VHR participants (p<0.0001).** *GDF-15, TRAIL-R2, MMP7, TNFα, MMP12, UPAR, IL-6, REN and IL-8 had an AUC over 0.8 suggesting a superior specificity and sensitivity compared to the other measured proteins. AUC values were calculated by a Receiver Operating Characteristic Curve (ROC). GDF: Growth/differentiation factor 15; TRAIL-R2: TNF related apoptosis-inducing ligand receptor 2; MMP7: Matrix metalloproteinase 7; TNFα: Tumour necrosis factor alpha; MMP12: matrix metalloproteinase 12; UPAR: urokinase receptor; IL-6: Interleukin 6; REN: Renin; IL-8: Interleukin 8*

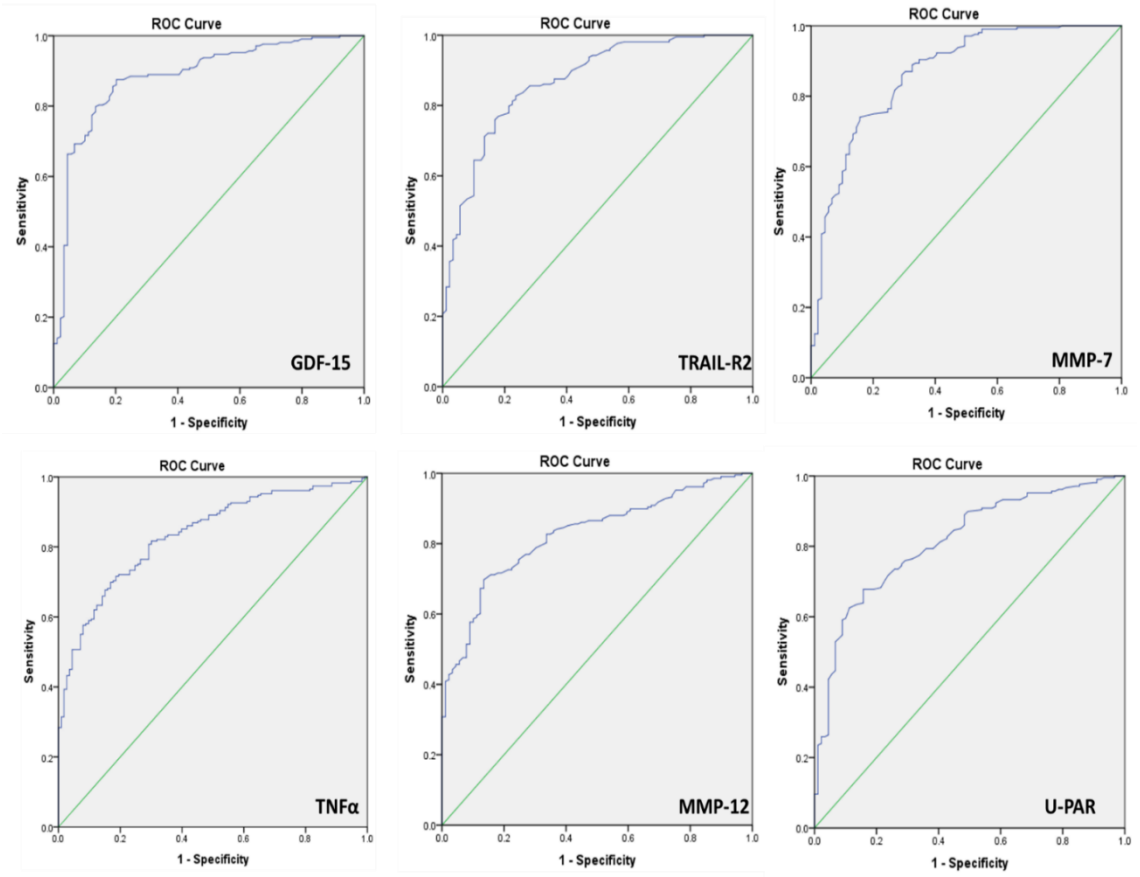


***Figure 4:* ROC curves for GDF-12, TRAIL-2, MMP7, TNFα, MMP-12 and U-PAR protein levels to assess sensitivity and specificity of the used assays in differentiating between VHR and non-VHR individuals**. *GDF-15 Growth/differentiation factor 15; TRAIL-R2: TNF related apoptosis-inducing ligand receptor 2; MMP-7: Matrix metalloproteinase-7; TNFα: Tumour necrosis factor alpha; MMP12: Matrix metalloproteinase-12; UPAR: Urokinase receptor; ROC: Receiver operating curve.*

8.3.7 Strengths and Limitations

Refer to **section 3.4.3 in Chapter 3** and **section 7.4.3 in Chapter 7.**

8.5 Conclusion

In conclusion, this study highlights the importance of evaluating different experimental conditions and measurement methods during biomarker development. This process should take place in parallel to the assessment of the clinical value of any potential marker. This will help uncover any experimental or technical problems that normally challenge the implementation of any new biomarker into clinical practice.

Chapter 9

General Discussion and Future Work

9.1 Major Findings

The present study highlights the role of a multimarker panel in improving cardiovascular risk assessment and in further stratifying individuals at very high risk of cardiovascular events. Such panels, once optimised, should be evaluated in large prospective longitudinal studies to validate their clinical value and assess their introduction into clinical practice and their value in providing a unique proteomic disease signature to each susceptible individual. In addition, this work provides further understanding of the biology and the mechanism of action of some of the investigated biomarkers. Some of the major findings of the present study are highlighted below:

TNFα Pathway: Following a Deductive Method in Biomarker Research

- TACE and TIMP3 are potential therapeutic targets in CVD and may have promising potential as CVD biomarkers.
- TNFR1 is an acute phase marker for primary and secondary cardiovascular events.
- TNFR1 and TNFR2 are specific markers for CVD patients with comorbidities such as diabetes, heart and renal failure.
- A panel combining TNFα, TNFR1 and TNFR2 could have potential in predicting major adverse cardiovascular event (MACE).

Proteomic Analysis: Following an Inductive Method in Biomarker Research

<u>Overall Findings</u>

- Multimarker panels have a superior value compared to single marker panels.
- Multimarker panels need to be optimised to represent proteins from different inflammatory pathways in CVD.

- The clinical validation of such panels needs to be done in parallel to the collection of follow-up data and blood samples from individuals in longitudinal studies.
- In order to guide therapy and management, it is possible to further stratify very high risk CVD individuals in high and low risk clusters using protein levels of potential markers.

Specific Findings

- MMP7, LOX-1, GLO1, MMP12, CTSD, REN, GDF-15, CNTN1, TRAP, hOSCAR, CEACAM8, PAAPA, TNFRSF11A, PON3, IL-6, IL-8, INF-γ and IL-1β are potential predictors of cardiovascular risk.
- ADAMTS-13, OPN, VEGF-D, Protein AMBP, BOC, PD-L2 and TRAP are promising markers for primary cardiovascular events.
- TNFSF13B, IL18-BP, TNFRSF11A, BOC, IFN-γ, IL-12p70, IL-8 and EGFR characterised patients admitted for MACE.
- IL-6, ST2, IL4RA, IL1RT1, IL1RT2, PDL2, ADM, THBS2, CCL16, RARRES2, GDF15, PI3 and SPON1 CCL22, ADAMTS13 and IDUA are potential markers for follow-up MACE.
- KIM1, IL-1RT1, Gal-3, REN, VSIG2, FABP4, GIF, IL-6, PON3, HO-1 identified VHR patients with diabetes.
- TRAIL-R2, FGF-21, ADM, TNFRS11A, INF-γ, IL-6, CTRC and SRC characterised VHR patients with heart failure.
- GDF-15, TRAIL-R2, MMP7, MMP12, U-PAR demonstrated a high sensitivity and specificity.

9.2 General Discussion

9.2.1 The need for better prediction tools

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide and even with major advances in medical and clinical management, CVD prevalence is increasing with 17.5 million global deaths in 2012 (1). In clinical practice, identifying individuals at risk of CVD has improved with the introduction of risk scores which are based on the most common CVD risk factors such as total cholesterol levels, diastolic blood pressure, diabetes, family history and smoking. Therefore, managing those risk factors could possibly delay CVD onset but won't completely avoid it. Additionally, troponin measurement has ameliorated the diagnosis of patients with an acute coronary syndrome (ACS). However, troponins are also known to be elevated in other inflammatory conditions (such as heart failure, tachyarrhythmias, myocarditis, hypertensive emergencies, Takotsubo cardiomyopathies etc.) which might delay an accurate diagnosis and the initiation of the appropriate therapy. On the other hand, even with optimal medical and clinical management, individuals with established CVD and a previous ACS are at high risk of major adverse cardiovascular events (MACE) (397). Hence, despite improving early CVD detection, controlling the risk factors and treating patients at risk of MACE, CVD management remains far from ideal.

9.2.2 Identifying patients at very high risk of cardiovascular events

In this present work, using the European SCORE risk chart to assign a score for each recruited participant, we explored whether this traditional risk score can be improved

by measuring promising novel biomarkers from the tumour necrosis factor alpha (TNFα) pathway as well as other inflammatory pathways.

TNFα is shed by tumour necrosis factor alpha converting enzyme (TACE) which is also known to shed several inflammatory markers known to be involved in the initiation and progression of atherosclerosis including TNFR1 and TNFR2 (220). Nevertheless, exploring proteins from this pathway as potential CVD biomarkers hasn't been assessed before.

On the other hand, in light of major advances in proteomics which are facilitating the discovery of potential biomarkers and novel therapeutic targets, we explored a number of proteins known to be involved in immunity, atherosclerosis, plaque rupture and thrombosis in order to assess their role as potential CVD biomarkers.

9.2.2.1 TNFα pathway in CVD

TNFα plasma levels and *TACE* gene expression were higher in very high risk (VHR) compared to non-VHR participants. TNFα plasma levels have been linked to CVD risk for over a decade (181) and *TACE* gene expression was previously shown to be increased in small cohorts of patients with acute myocardial infarction (MI) (170,171). However, *TACE* gene expression has not been measured before in a large number of individuals at various levels of cardiovascular risk. In addition, both TNFα receptors, tumour necrosis factor alpha receptor 1 (TNFR1) and 2 (TNFR2) plasma levels were higher in VHR compared to non-VHR participants. The present work is novel as levels of TNFR1 and TNFR2 have not been previously compared across individuals at various levels of cardiovascular risk. Both soluble receptors have only been associated with the presence of CVD co-morbidities such as renal disease, heart failure and diabetes (206,207) in VHR

individuals. In is noteworthy that TNFα, *TACE* gene expression and TNFR1 levels were sensitive enough in identifying risk as levels increased with increasing cardiovascular risk in parallel with the assigned SCORE risk.

The measurement of TNFR2 to TNFR1 cell membrane protein ratio in CVD is novel. TNFR1 receptor is a major initiator of inflammation causing endothelial cell dysfunction whereas TNFR2 receptor has more favourable effects by activating angiogenic and survival pathways (209,210). In this present work, TNFR2 to TNFR1 cell membrane protein ratio was lower in VHR compared to non-VHR patients suggesting that the balance between those receptors is more likely to shift towards a pro-inflammatory state which might be similar to what is observed in endothelial cells.

For the first time, TACE plasma levels were measured in CVD individuals highlighting a trend where levels were lower in VHR compared to non-VHR participants. The detection of TACE protein in the plasma highlights the presence of a mechanism behind its release into the bloodstream that requires further investigation. TACE is known to be a membrane bound protein whose presence on the cell surface is essential for an effective cleavage of substrates (151,152). Some studies however, have reported the presence of an active form of TACE protein in the plasma (176,177) where its activity was measured using a Fluorescence Resonance Energy Transfer assay. TACE has also been reported to be present on microparticles of platelet and endothelial origin (175,176). Nevertheless, whether TACE can effectively cleave distant substrates *in vivo* is unknown. This study also showed that cell membrane bound TACE protein levels were higher in VHR compared to non-VHR participants, which has not been explored previously. In summary, in contrast to the presence of low plasma TACE levels, VHR individuals have an increase in *TACE* gene expression and TACE cell membrane bound

levels. This could therefore lead to increased shedding of inflammatory transmembrane proteins and a pro-inflammatory state.

Moreover, this data is in line with a recent study that provided evidence that TACE is a good target for biological therapeutics. A bi-specific inhibitor, that targets cell membrane bound TACE and a pro-inflammatory cytokine, was able to increase its concentration at the cell surface and reduce inflammation in irritable bowel disease (202). The efficacy of such a promising therapeutical model should be tested in CVD. However, recent evidence has shown that TACE targeting needs to be cell specific and needs to take into account the stage of atherosclerosis (200).

Metalloproteinase inhibitor 3 (TIMP3) is the only known endogenous TACE inhibitor and *TIMP3* mRNA levels were lower in VHR compared to non-VHR participants. This suggests that, in contrast to an increase in *TACE* expression in individuals with CVD, *TIMP3* gene expression was decreased. As a result, TIMP3 fails to effectively inhibit TACE which would subsequently cleave a higher number of inflammatory transmembrane proteins. Plasma TIMP3 to cell membrane bound TIMP3 protein ratio was higher in VHR compared to non VHR patients indicating that in VHR individuals, a higher proportion of TIMP3 is released into the plasma compared to the amount of TIMP3 retained on the cell surface. TIMP3 retained on the cell surface is known to be associated with TACE to form dimer structures that inhibit TACE activity and substrate shedding (235,236). A lower proportion of TIMP3 on the cell surface results in an insufficient TACE inhibition which was also reflected by the cell membrane bound TIMP3 to TACE protein ratio which was significantly lower in VHR participants. When TACE is ineffectively inhibited by TIMP3, it shifts into its monomer active form, and subsequently cleaves a higher number of transmembrane proteins aggravating local and systemic inflammation (235). Previous

studies have shown that a loss of *TIMP3* increased the risk of atherosclerosis in ApoE null mice (233).

This provides further evidence of the role of TACE in CVD where its gene and protein expression seems to be increased while its endogenous inhibitor, TIMP3, fails to effectively inhibit its proteolytic activity leading to a pro-inflammatory state. Recent work has suggested the possibility of adopting a TIMP3 based therapy to offer cardio protectivity (234) and this needs further investigation.

9.2.2.2 Multimarker approach: TNFα inflammatory panel

Finally, the value of a multimarker *versus* a single marker approach in assessing an individual's cardiovascular risk was highlighted by the panel we tested which combined TNFα, TNFR1 and TNFR2 plasma levels in addition to *TACE* gene expression. In a binary logistic regression model, this 4-marker panel was able to collectively predict VHR/non-VHR group membership in 82.5% of the cases ($p<0.0001$) as opposed to 76.9% for TNFα, 74.7% for TNFR1, 67.7% for TNFR2 and 67.8% for *TACE* gene expression when all of these variables were entered in the model separately.

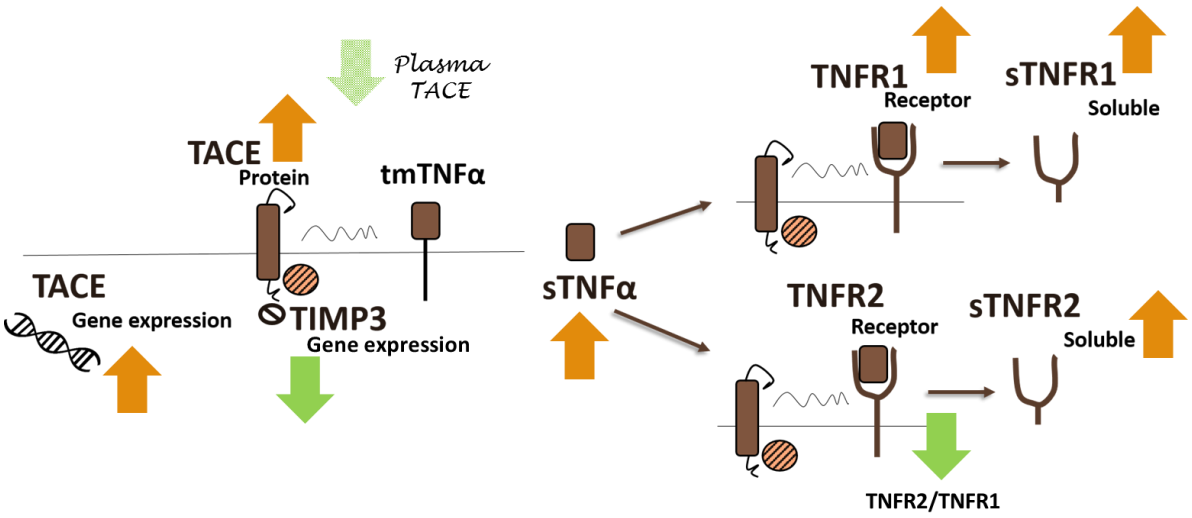


***Figure 1:* Increased or decreased levels of proteins from the TNFα inflammatory pathway in individuals at very high risk (VHR) of CVD.** *In patients at very high risk of CVD,* TACE *gene expression and TACE cell membrane bound levels were higher in VHR vs. non-VHR individuals. However, TACE plasma protein levels showed a trend where levels were lower in VHR vs. non-VHR individuals.* TIMP3*, TACE endogenous inhibitor, gene expression was lower in VHR vs. non-VHR individuals. TACE is known to cleave tmTNFα releasing sTNFα which plasma levels were higher in VHR vs. non-VHR individuals. TACE is also known to cleave TNFR1 and TNFR2 releasing sTNFR1 and sTNFR2 respectively. sTNFR1 and sTNFR2 plasma levels were higher in VHR vs. non-VHR individuals. TNFR2 to TNFR1 cell membrane bound ratio was lower in VHR vs. non-VHR individuals.*
TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: Tissue inhibitor of metalloproteinase 3; tmTNFα: transmembrane tumour necrosis factor alpha; sTNFα: soluble tumour necrosis factor alpha; TNFR1: tumour necrosis factor alpha receptor 1; TNFR2: tumour necrosis factor alpha receptor 2

9.2.2.3 Exploring new pathways in CVD: A proteomic approach

It is now accepted that CVD frequently can occur concomitant with other co-morbidities such as diabetes, arthritis, depression, renal disease and cancer which all share common biological and immune pathways. In this present work, 53.3% of VHR individuals presented with a comorbidity (diabetes, arthritis or depression). Therefore, in CVD biomarker research, the present consensus is shifting towards a multimarker approach as it is becoming clearer that the complexity of CVD is unlikely to be captured by a single marker. A proteomic approach that consisted of analysing 193 proteins known to be involved in atherosclerosis, plaque rupture, thrombosis, as well as the inflammatory and

immune response, identified novel proteins which role in CVD risk assessment hasn't been previously described. In addition, well-known associations between potential biomarkers and cardiovascular risk were highlighted providing further evidence of their promising role in CVD risk assessment.

Matrix metalloproteinase-7 (MMP7), Lectin-like oxidized LDL receptor 1 (LOX-1), Lactoylglutathione lyase (GLO1), Matrix metalloproteinase-12 (MMP12), Cathepsin D (CTSD), Renin (REN), Growth/differentiation factor 15 (GDF-15), Contactin-1 (CNTN1), Thrombospondin-related adhesive protein (TRAP), Osteoclast-associated immunoglobulin-like receptor (hOSCAR), Carcinoembryonic anti-genrelated cell adhesion molecule 8 (CEACAM8), Pappalysin-1 (PAAPA), Tumour necrosis factor receptor superfamily member 11A (TNFRSF11A), Paraoxonase (PON3), Interleukin-6 (IL-6), Interleukin 8 (IL-8), Interferon gamma (INF-γ) and interleukin 1 β (IL-1β) protein levels were potential predictors of cardiovascular risk. In addition, GDF-15, MMP7, MMP12, REN, IL-6 and IL-8 measurement assays had a good specificity and sensitivity which are important qualities for novel candidate biomarkers for future development (137).

Our results are in line with previous reports on GDF-15 as new biomarker for stable coronary artery disease (CAD), one of the major forms of CVD, acute coronary syndrome (ACS) and heart failure (308). In addition, our study showed that GDF-15 has a good sensitivity and specificity. Among the other markers that demonstrated good sensitivity and specificity in this study, we cite MMP7 which is known to contribute to plaque instability in atherosclerosis (297) and MMP12 which has been suggested as a candidate molecule for the prevention and treatment of cardiometabolic diseases (302). In addition, REN plasma activity has been recently linked to increased risk of MACE and congestive heart failure in patients with high systolic blood pressure (307). Prospective

future studies are needed to evaluate whether these markers, alone or as part of a multimarker strategy, can improve current risk prediction tools and support therapeutic management of patients with CVD.

Other proteins that were associated with cardiovascular risk but showed lower sensitivity and specificity include LOX-1, which is known to be a marker for atherosclerosis related events (298) and GLO1 where polymorphisms in the *GLO1* gene have been associated with vascular diseases (301). In addition, CTSD is known to have a role in MACE occurrence (305) whereas paraoxonases enzymes are shown to contribute to CVD especially through their involvement in the lipid metabolism (309). Moreover, the role of CNTN1 in predicting new CVD onset has also been previously highlighted (122). On the other hand, IL-6 levels have been linked to cardiovascular risk for over a decade (255,256) whereas IL-8 is known to be involved in maintaining an inflammatory environment surrounding the plaque (245) and levels were shown to be higher in unstable CAD (246). IFN-γ is known to play an important role in atherosclerosis and plaque stability (254) however, IFN-γ plasma levels haven't been measured yet in patients at different levels of cardiovascular risk and have demonstrated a controversial role in CVD within this project. IL-1β has long been linked to atherothrombotic disease and plaque destabilisation as well as adverse remodelling after an MI (264) and in the present study, *IL1B* gene expression was higher in VHR compared to non-VHR participants. IL-1β has proven to be an effective therapeutical target in CAD as the ongoing Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial is showing promising results (263).

This study also highlights a novel role of several proteins such as TRAP, hOSCAR, CEACAM8, PAPPA, TNFRSF11A and PON3 in CVD and their role should be further investigated in addition to their evaluation as potential CVD biomarkers.

9.2.3 Identifying CVD individuals at risk of MACE

As VHR individuals are at high risk of secondary events despite optimal revascularisation strategies and medical management, the aim of this present investigation was to also explore whether proteins from the TNFα inflammatory pathway as well as proteins from other pathways involved in atherosclerosis, plaque rupture, thrombosis and immunity allow a sub-classification of VHR individuals in terms of comorbidities as well as MACE risk.

9.2.3.1 TNFα pathway in differentiating between VHR patients

By exploring the TNFα pathway in VHR patients, we demonstrated that TNFα plasma levels remain high in VHR patients with previous MI or previous percutaneous coronary intervention (PCI) despite medical and clinical management. Persistent elevated TNFα plasma levels post-MI have been linked to an increased MACE risk (181). Previous reports have demonstrated the role of TNFα in pre-conditioning the heart against an inflammatory environment (398) following a myocardial infarction. This needs further exploriation in CVD individuals.

TACE gene expression was lower in VHR patients with a previous PCI who were also on statin, antiplatelet and antihypertensive therapy. TACE is known to regulate angiogenesis in cardiomyocytes following MI and, when upregulated within the normal range, is suggested to provide protective effects post-MI (194). Therefore, *TACE*

downregulation in patients with previous PCI could be associated with adverse outcomes. The effect of medical and clinical management on *TACE* gene expression needs further understanding.

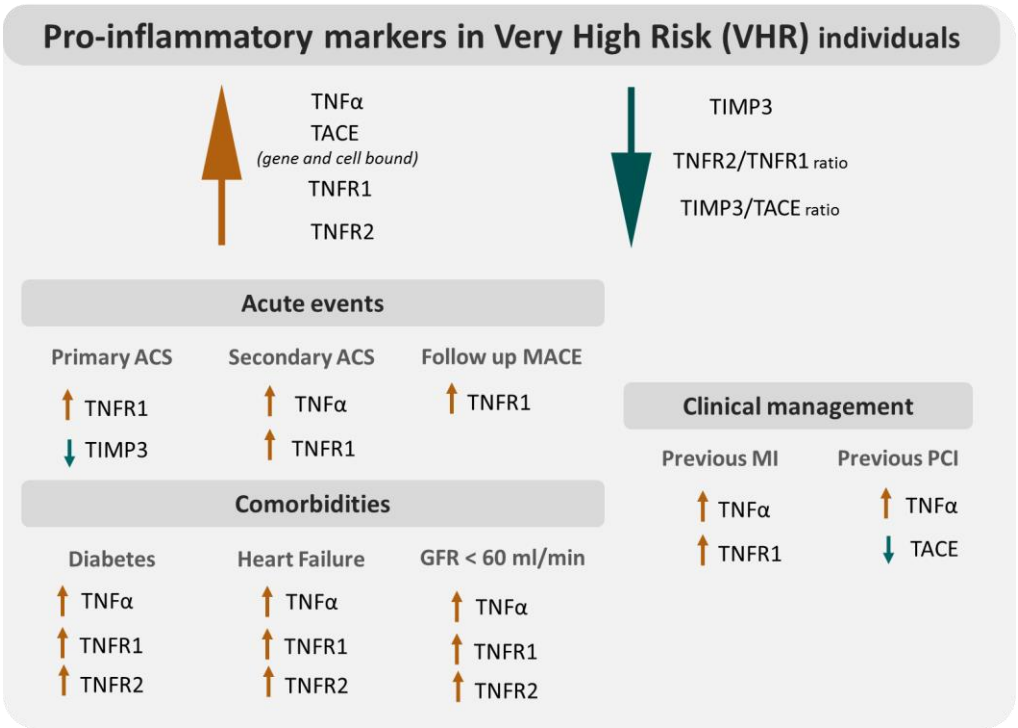
Furthermore, it appears that after a PCI, despite a decrease in *TACE* gene expression, TNFα plasma levels are increased. This could be due to different activators that, when present, are known to enhance and modulate TNFα shedding (187) and to different sheddase, such as ADAM10 (188), MMP7 (189) or MMP13 (190) that have been suggested to be involved in TNFα shedding. Furthermore, circulating TNFα might be captured by circulating TNFR1 and TNFR2 soluble receptors as suggested by Van Zee *et al*. and therefore, TNFα might not be biologically active (191).

In this current study, TNFR1 plasma levels were higher in participants admitted for acute events with higher levels in ACS patients admitted for their primary or secondary cardiac event (MACE) which suggests that TNFR1 could be a potential marker for acute cardiovascular events. TNFR1 and TNFR2 plasma levels were higher in VHR patients with diabetes, heart failure and renal disease indicating that levels tend to be even higher when certain comorbidities are present along with CVD as reported previously (206,207,224). TNFR1 plasma levels were also increased in VHR patients with depression as previously shown (399).

Plasma levels of TIMP3, which is known to regulate hypertension (231), were higher in VHR patients on antihypertensive therapy compared to VHR patients not on antihypertensive therapy. *TIMP3* was also downregulated in ACS-VHR patients compared to elective percutaneous intervention (ELEC-VHR) patients showing that close to an ACS, *TIMP3* gene expression is particularly decreased. Down-regulation of *TIMP3* has been previously shown to increase TACE expression and TNFα production by

placental trophoblast cells (232) providing evidence that a decrease in *TIMP3* gene expression could be accompanied by an over expression of TACE and an aggravation of local and systemic inflammation and the occurrence of acute cardiovascular events. In addition, *TIMP3* expression has been previously shown to be particulary low in stable plaque and high in vulnerable plaques (400) providing further evidence of the importantce of TIMP3 in CVD.

Furthermore, the ability of TNFα, TACE, TNFR1 and TNFR2 in further stratifying VHR individuals proved to be superior to CRP. Our results showed that CRP plasma levels were not significantly different between ACS-VHR and ELEC-VHR participants admitted for a first or recurrent event. Furthermore, CRP plasma levels were not able to identify VHR participants with previous MI or PCI or VHR participants with comorbidities such as diabetes, heart failure and renal failure.



<u>Figure 2:</u> Levels of proteins from the TNFα inflammatory pathway can differentiate between sub-groups of very high risk (VHR) of CVD. *In participants at very high risk (VHR) of CVD, TNFα plasma levels,* TACE *gene and protein expression as well as TNFR1 and TNFR2 plasma levels were higher vs. non-VHR. TIMP3 gene expression and TNFR2 to TNFR1 as well*

as TIMP3 to TACE cell membrane bound ratios were lower in VHR vs. non-VHR participants. TNFα plasma levels were higher in VHR individuals admitted for a secondary event, in VHR diabetic participants, VHR participants with heart and renal failure, in VHR participants with previous MI and a previous PCI and in VHR participants on drug therapy. TNFR1 plasma levels were higher in VHR individuals admitted for a primary or a secondary event, in follow-up participants who developed MACE, in VHR diabetic participants, VHR participants with heart and renal failure, in VHR participants with previous MI and in VHR participants on drug therapy. TNFR2 plasma levels were higher in VHR diabetic participants, VHR participants with heart and renal failure and in VHR participants on drug therapy. TIMP3 gene expression was lower in ACS-VHR vs. ELEC-VHR participants. TACE gene expression was lower in VHR participants with a previous PCI. ACS: Acute coronary syndrome; GFR: glomerular filtration rate; MACE: Major adverse cardiovascular events; MI: Myocardial infarction; PCI: Percutaneous coronary intervention; TACE: Tumour necrosis factor alpha converting enzyme; TIMP3: matrix metalloprotease inhibitor 3; tmTNFα: Transmembrane tumour necrosis factor alpha; sTNFα: soluble tumour necrosis factor alpha; TNFR1: tumour necrosis factor alpha receptor 1; TNFR2: tumour necrosis factor alpha receptor 2

9.2.3.2 A proteomic approach in stratifying VHR patients

The proteomic approach followed in this study identified a set of proteins that differentiated between VHR participants in terms of their cardiovascular history and associated comorbidities. Several markers were specific to the onset of ACS in participants with previous or no previous CVD history, while others were related to clinical and medical management. Moreover, some markers were specific to VHR participants with diabetes or heart failure highlighting the need to further understand the underlying interactions and pathways behind CVD and its associated comorbidities which could lead to the discovery of potentially new therapeutic targets. The proteins that were able to substratify the VHR cohort are summarised in figure 3.

9.2.3.2.1 Markers for acute cardiovascular events

Among the proteins that were able to differentiate between ACS-VHR and ELEC-VHR participants, there was little evidence for the role of Macrophage receptor (MARCO), C-C motif chemokine 15 (CCL15), Polymercic immunoglobulin receptor (PIgR) and Cell

Adhesion Associated, Oncogene Regulated (BOC) in CVD and their role in identifying patients with acute cardiovascular events should be explored further.

The analysis of ACS-VHR and ELEC-VHR with no previous CVD history provided further evidence of the role of the importance of A disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS-13) (312), Osteopontin (OPN) (322) and Protein AMBP (323) as botential CVD biomarkes. However, the role of programmed death ligand 2 (PDL2), BOC and Tartrate resistance acid phosphatase 5 (TRAP) in CVD hasn't been previously outlined. Such proteins should be tested further as biomarkers for primary cardiovascular events.

When considering proteins associated with MACE as opposed to a first cardiovascular event, novel and known associations were highlighted. Among these assocaitons, IL-12 levels have been previously shown to be related to adverse outcomes in patients with STEMI (272) while EGFR activation has been implicated in blood pressure regulation, endothelial dysfunction, atherogenesis, and cardiac remodelling (325). Nevertheless, Tumour necrosis factor ligand superfamily member 13B (TNFSF13B), receptor superfamily member 11A (TNFRSF11A) and BOC haven't been measured in CVD patients yet. Such markers hold a potential role in determining individuals at higher risk of secondary events.

It appears that BOC is a marker for ACS events and hasn't been investigated previously in CVD. BOC has been reported do be involved in the differentiation of myogenic cells (401). However, the present results are in favour of its role as a potential diagnostic biomarker in CVD.

9.2.3.2.2 Markers associated with clinical management and comorbidities in CVD

Among the protein levels which differentiated between VHR participants with previous MI, PCI or coronary artery bypass grafting (CABG) compared to those with no previous MI, PCI or CABG, Prostasin (PRSS8) has been suggested as a potential gene for hypertension (326) whereas Interleukin 16 (IL16) has been shown to promote cardiac fibrosis and myocardial stiffening in heart failure patients (327). Moreover, LDL receptor (LDLR) plasma levels have ben previously shown increase following a statin therapy (402). However, there is no evidence of the role of V-set and immunoglobulin domain containing protein 2 (VSIG2) in CVD which should be further explored to evaluate the mechanisms in place after the initiation of a clinical or a medical treatment in CVD.

In relation to VHR individuals who also had diabetes, the proteins that were mostly highlighted were Kidney injury molecule 1 (previously TIM and now as KIM1) which has been linked to renal disease in diabetic patients (330) and cardiovascular risk (331), Galectin 3 (Gal-3) (334,336) and Renin (REN) (307,338) which have also been studied in diabetic patients at high risk of CVD. However, the role of VSIG2 and Gastric intrinsic factor (GIF) in CVD and diabetes is less clear. These proteins could reflect the underlying pathways behind the interaction between CVD and diabetes and could prove to be potential therapeutic targets if studied in larger cohorts.

When considering the proteins characterised VHR individuals with heart failure, Fibroblast growth factor 21 (FGF-21) has been reported to be associated with cardiovascular risk (342) and heart failure (343) whereas Adrenomedullin (ADM), which is known to regulate vasodilatation and NO production, has been suggested to play a protective role in CVD and heart failure (344). However, the role of Tumour necrosis

factor receptor superfamily member 11A (TNFRS11A) and Chymotrypsin C (CTRC) in CVD and heart failure is less clear. Such proteins could hold a promising value in assessing CVD patients at high risk of heart failure and could prove to be potential therapeutic targets if measured in larger cohorts.

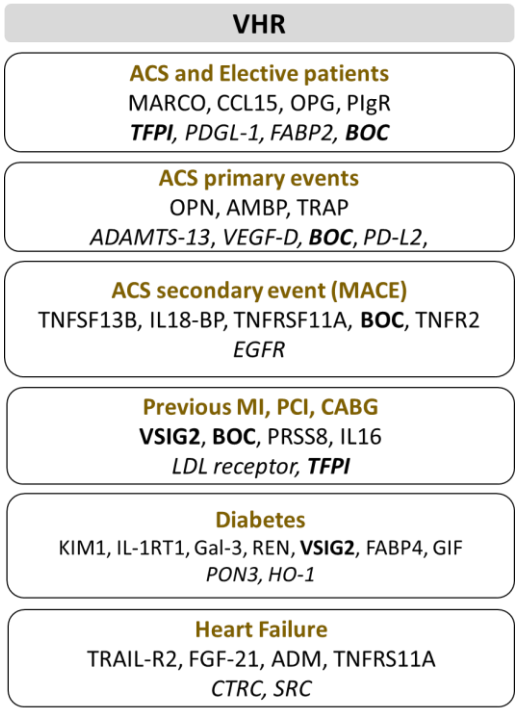


***Figure 3:* Summary diagram of a proteomic analysis: Potential proteins to further stratify the VHR cohort.** *Proteins in bold are able to differentiate between more than two VHR risk groups. Proteins in italic are lower in the first group compared to the second. ACS: acute coronary syndrome; CABG: coronary artery bypass grafting; MACE: major adverse cardiovascular events; MI: myocardial infarction; PCI: percutaneous coronary intervention; VHR: very high risk.*

9.2.3.3 Biomarkers predicting MACE risk

Biomarkers associated with cardiovascular risk, and the development of a first cardiovascular event, in patients with no previous cardiac history have been intensively studied. Nevertheless, biomarkers associated with the development of secondary cardiovascular events need to be further investigated because at present, there is no

effective test that can accurately predict which patient will develop MACE. Taking forward the previously measured inflammatory markers, we aimed to assess their association with MACE risk. A summary of this section is provided in figure 4.

Among the highlighted proteins that were associated with MACE risk, TNFR1 was associated with MACE risk within one year of admission showing a trend where levels were particularly higher after 6 months of admission whereas IL-6 plasma levels were associated with MACE risk within one year and specifically within 6 months after admission. These results are in line with previous reports that highlighted an association between TNFR1 levels and death as well as heart failure onset in patients post-MI (222) and between IL-6 levels and future MACE (266,267,271). Among the other markers that demonstrated to be associated with MACE risk, IL-1RL1/ST2 has been previously reported to be a marker for ACS and acute heart failure (357) and is also reported to be marker for cardiac stress and was strongly associated with the development of heart failure post-ACS in the MELRIN-TIMI 36 trial (358). IL-1RT1 has been suggested as a novel inflammatory marker for CVD (361) which was also involved in heart remodelling post-MI (333). ADM has been shown to be a powerful independent predictor of future cardiovascular events in high-risk patients with a predictive value superior to that of high sensitivity C reactive protein or adiponectin (362). THBS2 has been linked to the development of heart failure in patients with CAD (366) whereas CCL16 has been recently associated with CAD risk in postmenopausal women (368), however, their value in MACE prediction hasn't been assessed yet. RARRES2 is a recently discovered adipokine that has been linked to inflammation and increased oxidative stress in obese, CVD and ACS patients (369–371). GDF-15 has been recently suggested as a biomarker for CVD and MACE (373) and is shown to have a unique capacity in capturing CVD

development, progression, and prognosis (374). Pl3 is an endogenous inhibitor of neutrophil-derived elastases which presented an anti-inflammatory activity in pre-clinical models of inflammatory vascular injury (376). On the other hand, our results are in line with previous reports regarding ADAMTS13 which low levels were shown to contribute to haematological and cardiovascular disorders (312) and severe deficiency in ADAMTS13 results in a clotting disorder known as thrombotic thrombocytopenic purpura (381). However, there is little evidence of the role of interleukin 1 receptor 2 (IL1RT2), PDL2, alpha-L-iduronidase (IDUA) and C-C motif chemokine 22 (CCL22) in CAD and MACE prediction. Further work is needed in order to evaluate the clinical value of such proteins in MACE risk assessment.

A logistic regression model combining all the previously mentioned proteins was able to significantly predict follow-up MACE in 94.2% of the cases ($p<0.0001$). This useful model highlights the importance of a multimarker approach not only in CVD primary prevention but also in secondary prevention since a model combining several clinically relevant markers has a better prediction value compared to a single marker model. Therefore, the several limitations that characterise the current CVD biomarkers can be overcome by the use of a multimarker approach (384).

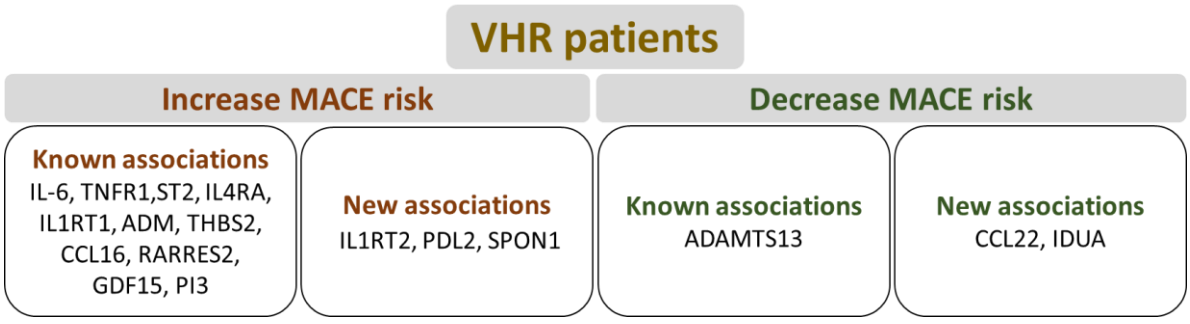


***Figure 4:* Previously established and novel associations between inflammatory proteins and MACE risk.** *MACE: major adverse cardiovascular events; VHR: very high risk.*

In addition, the definition of MACE in this study was restricted to recurrent ACS, stroke TIA and death. However, several studies include HF and atrial fibrillation (AF) (203,204) in the definition for MACE as well. By including HF and AF in the MACE definition, the total number of patients who develop MACE within a year of admission increases to 13.5% as opposed to 7.0% when HF and AF are not included.

9.2.3.4 Cluster analysis: a tool to further stratify VHR patients

Cluster analysis is proving to be an effective technique in patient stratification and especially in further stratifying very high risk patients (403). Recently, using a cluster analysis, it was possible to determine biomarker signatures of aging (404) as well as define novel classes of diabtetes (405). Using the proteins that were able to differentiate between VHR individuals who were admitted for a primary or a secondary cardiovascular event, and those who developed MACE over time, we aimed to further stratify VHR individuals using cluster analysis. The VHR cohort formed two clusters with cluster 2 grouping participants with higher levels of most of the inflammatory markers compared to cluster 1. When analysed in relation to other characteristics, cluster 2 grouped older participants, participants who were likely to be admitted for an ACS and to be admitted for MACE. Cluster 2 also grouped participants who were more likely to be diabetic, to have had a previous MI or PCI, and to be on medication. The proteins that contributed majorly to the cluster classification were: Phospholipase C (PLC), Trefoil factor 3 (TFF3), Tumour necrosis factor alpha receptor 1 (TNFR1) and 2 (TNFR2), Growth/differentiation factor 15 (GDF15), Adrenomedullin (ADM), TNF Receptor Superfamily Member 14 (TNFRSF14), Urokinase receptor (UPAR) and Lymphotoxin Beta Receptor (LTBR) which were all higher in cluster 2. The expression and activity of PLC were previously reported to be elevated in cardiomyocytes under pathological conditions such as ischemia and hypertrophy (406) whereas ADM was proposed to be a biomarker of prognosis and survival in patients with coronary artery disease or heart failure (344). TNFRSF14 is a player in atherogenesis by inducing proinflammatory cytokines and matrix metalloproteinases (348). UPAR has been intensively studied in chronic kidney disease (CKD) and was been suggested as a biomarker for CVD in patients

with (CKD) (349), in addition, LTBR appears to play a role in macrophage-driven inflammation in atherosclerotic lesions (350). However, the role of TFF3 in CVD is limited. These results show that the previously mentioned proteins can further stratify VHR patients and could be part of a panel that would indicate which VHR patients are at higher risk of cardiovascular events.

9.2.4 Obstacles in translating biomarker research into clinical practice

In the past decade, CVD management has improved with the implementation of risk scores charts that guide primary and secondary prevention. However, these risk scores have many limitations and leave ample room for improvement but could potentially be improved by the addition of biomarkers. However, the translation of biomarker research into clinical practice is proving to be very difficult with only two biomarkers currently used in CVD clinical practice (troponins and natriuretic peptides). Some of the major obstacles to this transition are listed below and some were encountered throughout this study.

9.2.4.1 Restricted number of recruited patients

As the current analytical methods for biomarker measurement (such as immunological, gene expression, flow cytometry and cell culture assays) require a considerable amount of time when it comes to sample processing and analysis, this limits the ability to obtain a quick result and therefore restricted the number of recruited patients in this present study. The solution to this problem lies in the development and validation of biomarker panel assays in the research laboratory prior to the assessment of their value in clinical practice. These assays should be adapted to point of care testing, generate reproducible

results, provide a high level of accuracy, specificity and sensitivity and be able to produce quickly interpretable results.

Another possible solution to increase the number of recruited patients in a study is by integrating research within the healthcare system. This would mean that every patient who attends the cardiology clinic is automatically asked to consent and volunteer to give a blood sample. This will make patient recruitment much more efficient and will strengthen and validate the prediction models associating biomarkers with cardiovascular outcomes.

9.2.4.2 Assessing the value of potential biomarkers by patient follow-up

The ideal way to validate the utility of novel biomarkers is by measuring them in the same patient over time and linking this biological measurement to any phenotypical and clinical data, which might also change over time, in the same patient. In this present study, all patients were contacted for to provide a follow-up blood sample, however, the number of patients who attended their appointment was very low. A multidisciplinary team involving researchers, clinicians and nurses is needed in order to facilitate the patient follow up process and the collection of follow-up data. However, this could be resolved by integrating research into the clinical practice where every patient attending a routine appointment actively participates in research progress by providing clinical and biological information throughout the different stages of his/her disease. This will provide valuable information and will allow biomarker research to progress at a higher speed. On the other hand, in the new era of personalised medicine, it is sometimes easy to forget the crucial role that patients play in moving the research forward. Patients need to be actively included in the study process and this could be

done by encouraging their interaction *via* smartphone applications or by organising public involvement events. It is essential for patients to understand their contribution to CVD research and the global impact that is resulting from their willing to donate a simple blood sample.

Other factors related to the development of the biomarker assay need to be investigated during the validation step. For instance, the stability of the biomarker needs to be assessed in addition to its specificity, sensitivity, negative and positive predictive values among other measurments. In addition, introducing a new biomarker panel into clinical practice needs to be cost-effective.

9.2.4.3 Translation of biomarker research into clinical practice

Translating biomarker research into clinical practice has proven to be very challenging over the years. One possible reason is that most of the clinical studies focused on one biomarker alone whereas the novel trend is by developing multiple markers. In addition, the value of these markers needs to be assessed longitudinally. Essentially, it is important to assess the improvement of risk prediction when the novel biomarker or multimarkers are added to the already existing risk scores. This should be evaluated by measurements such as c-statistics, the net reclassification improvement (NRI) and integrated discrimination improvement (IDI) indexes (99). In addition, novel prediction models and algorithms need to be developed in such a way to include known and novel markers in addition to phenotypical and clinical data. This will generate a much more accurate approach in relation to risk prediction.

9.2.4.4 Predicting risk to ultimatlty protect and prevent CVD

Predicting risk is of no value unless the patient can be protected. Much work is still needed in this area because the changes in biomarker levels reflecting an abnormal disease state do not as of yet direct changes in therapy or management. Nevertheless, using a novel multimarker approach to improve CVD risk prediction prompts the clinician and the patient to work together in order to decrease a high CVD risk when present or seek to maintain a low risk.

In addition, in order to assess the practicalities of introducing a new CVD risk prediction tool in primary prevention, a close collaboration needs to be organised with the general practitioners (GPs) as their role is crucial in indetifying patients that require further asssement and follow-up.

9.2.5 Conclusions and Future Work: The promise of personalised medicine is possible with multimarker models

Personalised medicine uses clinical measurements and parameters to implement a therapeutic strategy that is tailored to each individual. The concept of personalised medicine is becoming more of a reality with major advances in genetic research such as the international Hap-Map project and the Genome-Wide Association Studies (GWASs) as well as proteomic research that are revolutionising the field of diagnostics and therapies. Nevertheless, despite major technological advances and the ability to generate and interpret genomic and proteomic derived data, the impact of this progress on clinical practice is happening at a slow rate.

Alongside the advances in personalised medicine, the interest in biomarker discovery is increasing. Several studies have already investigated the role of several proteins

highlighted in this study in CVD. However, each study focused on one single marker alone linking it to a specific biological pathway, a specific clinical outcome or a specific CVD co-morbidity. A substantial amount of research linking proteins and polymorphisms to CVD already exists. The following step lies in developing multimarker panels that capture specific inflammatory pathways in CVD and optimised to identify acute events, recurrent events, risk of heart failure, risk of renal disease, or risk of diabetes. New bioinformatic tools and algorithms (407) combining such panels in a risk assessment model will facilitate their use in clinical practice. These markers could be added to the current cardiovascular risk prediction models and the present CVD biomarkers in order to overcome their limitations and provide a more accurate prediction of first or recurrent cardiovascular events.

It is important to remember that the multidisciplinary nature of personalised medicine will require harmonisation across different areas including changes in healthcare infrastructure, improvement of diagnostic tests and point of care testing and managing ethical and legal issues in relation to data security. Therefore, in order to initiate the bench to bedside transition, healthcare professionals, research committees, industrial representatives and legal authorities are required to collaborate and merge their efforts towards accomplishing one main goal which is improving patient's management by offering the best therapy to the right patient at the right time (Figure 5).

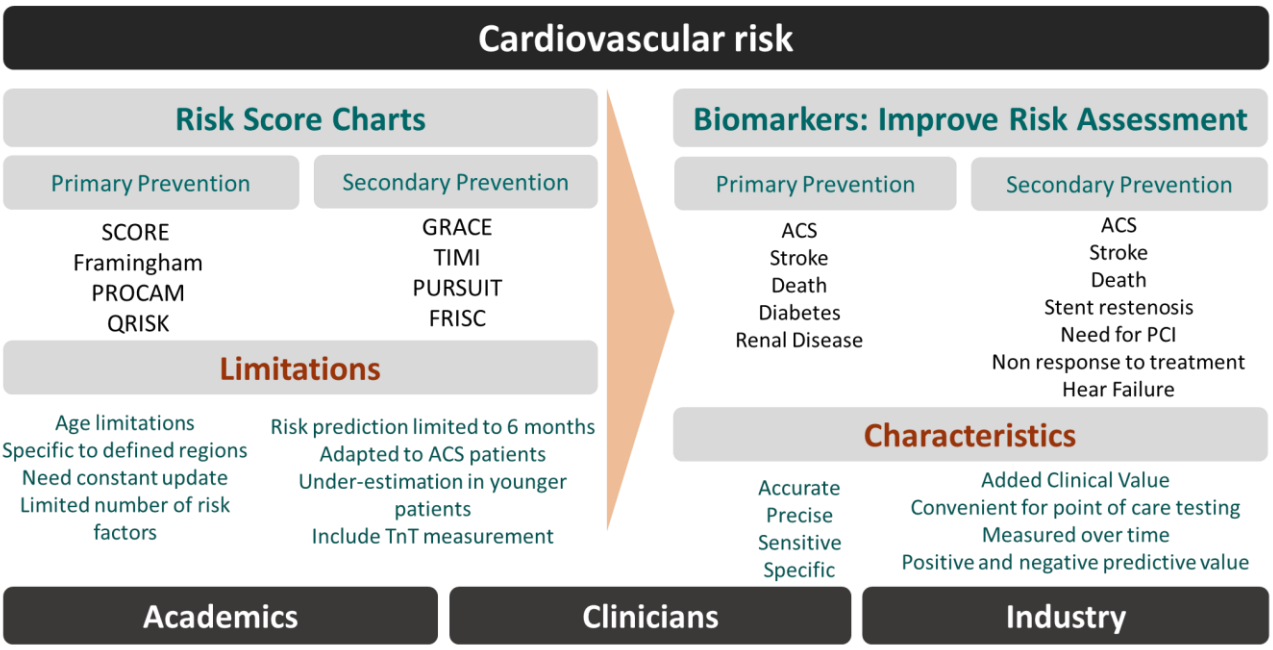


Figure 5: ***Improving Cardiovascular Risk Assessment by Incorporating Biomarkers to the Traditional Risk Scores.*** *Risk assessment score charts in cardiovascular disease (CVD) are based on the traditional risk factors and are used either for primary or secondary prevention. Those risk charts are known to have a lot of limitations. CVD risk assessment can be improved by the incorporation of biomarkers and can be used in primary or secondary prevention. The development and validation of biomarker panels is only possible with a close collaboration between academics, researchers and industries. ACS: Acute coronary syndrome; FRISC: fast revascularisation in instability in coronary disease; GRACE: Global Registry of Acute Coronary Events; PCI: Percutaneous coronary intervention; PROCAM = Prospective Cardiovascular Munster Study; PURSUIT: Platelet glycoprotein IIb/IIIa in Unstable angina: Receptor Suppression Using Integrilin; QRISK: based on the NICE guidelines on lipid modification; SCORE: Systematic COronary Risk Evaluation; TIMI: Thrombolysis In Myocardial Infarction.*

Appendices

I. Additional Findings

I.I Seasonal changes and biomarker levels

Background: The role of seasonal changes on inflammatory biomarker levels has been receiving a lot of interest recently (408) as the incidence of some diseases increases or decreases with seasonal changes. It has been found that during the cold seasons, the immune system is in a pro-inflammatory state (409,410). Therefore, the incidence of seasonal affective disorder (411), arthritis (412), blood pressure (413), cardiovascular disease (CVD) and respiratory morbidity and mortality (414) have been shown to occur more frequently in the cold compared to the warm seasons. The aim of this present study was to compare plasma protein levels of inflammatory and immune markers involved in the initiation and complications of CVD across different seasons.

Results: Recruited participants were all at very high risk (VHR; n=229) and have > 10% risk of cardiovascular death within 10 year. Tumour necrosis factor alpha (TNFα) plasma levels were the highest in winter ($p<0.05$) compared to the other seasons. Tumour necrosis factor alpha receptor 1 (TNFR1), interleukin 2 and interleukin 8 plasma levels were also the highest during the winter season ($p<0.01$; $p<0.01$ and $p<0.05$).

Discussion: Many factors such as temperature, physical activity, infections and food habits are known to increase the incidence of mortality and morbidity due to CVD during the winter season (415). Air pollution is also known to be higher during the cold seasons (416). In this present study, higher levels of TNFα, TNFR1, IL-2 and IL-8 during the cold seasons could be one of many other markers known to be associated with an increase in the incidence of CVD and cardiovascular events in the cold seasons (414). In addition, it has been shown that plasma levels of fibrinogen, cholesterol and catecholamine are

known to rise in winter (417) which could precipitate cardiovascular complications during the cold seasons.

Conclusion: Offering an intensive medical therapy combined with a change in lifestyle habits and the implementation of public health measures during cold seasons might help decrease the incidence of CVD events and complications.

I.II Endothelial dysfunction and cardiovascular disease

Background: The endothelium exerts a number of vaso-protective effects, such as vasodilation, suppression of smooth muscle cell growth, and inhibits inflammatory responses (418). Endothelial dysfunction is involved in atherosclerotic lesion formation and is characterised by an up-regulation of adhesion molecules and chemokines leading to leukocyte recruitment, an increase in cell permeability leading to low-density lipoprotein retention and oxidation, an induced vascular smooth muscle cell proliferation and migration (419). Thus, the endothelium is implicated in early lesion formation as well as the maintenance of a constant inflammatory environment around the lesion leading to complications and plaque rupture (420). Endothelial progenitor cells (EPC) are widely believed to be strong biomarkers of vascular risk. Endothelial colony forming cells (ECFCs), a subtype of EPCs, represent an endothelial cell type with angiogenic capacity, *de novo* blood vessel formation and vascular repair properties of the injured endothelium (421). ECFCs are normally known to be CD31+, CD105+, CD146+, CD45- and CD14- and can be obtained after culturing the peripheral mononuclear blood cells (PBMCs) in endothelium growth media (422).

Methods: In this present study, ECFCs were isolated from participants at very high risk (VHR) of CVD and participants at low risk (LR) of CVD. VHR participants had > 10% risk of cardiovascular death within 10 years whereas the LR participants had a <1% risk of

cardiovascular death within 10 years according to the SCORE risk chart (ESC guidelines). Different blood collection methods were tested to evaluate ECFCs formation *in vitro*. BD cell preparation tubes (CPT® - BD 362753) contained a liquid density medium and a gel barrier and required a centrifugation and two washing steps in order to collect the PBMCs. Histopaque®-1077 Ficoll extraction technique was also used as another method to obtain PBMCs where, in this case, the liquid density medium was added to the withdrawn blood, centrifuged and then PBMCs were washed twice before culture. An AutoMACS separation step was carried out in order to deplete the PBMCs (collected using the CPT® tubes) from CD45+ cells using CD45 antibodies coupled to magnetic beads. The collected PBMCs were then cultured as per the colony forming assay (CFA) protocol described in Martin-Ramirez et al (160).

Results and Discussion: A total of 14 VHR and 3 LR participants were recruited. 4 participants had their PBMCs isolated using the CPT® tubes, 5 using Histopaque®-1077 Ficoll extraction and 5 had their isolated PBMCs undergo a depletion of cells expressing the CD45 surface marker. After performing the colony forming assay for 3 weeks, the number of colonies were counted and are expressed in percentages (PBMCs were cultured in 96-well plates and each well with a formed colony was regarded as a positive well) (Table 1). After CD45 depletion ECFCs appeared much earlier at day 15 whereas the rest of the colonies appeared after day 20. Furthermore, the number of colonies that were obtained after CD45 depletion appeared to be higher than the ones obtained using the CPT® or using Histopaque®-1077 Ficoll extraction (Table 1). This could be due to the fact that CD45+ cells are more likely to secrete pro-inflammatory mediators that will delay the appearance of the ECFC colonies (423). In addition, the number of ECFC colonies obtained in VHR participants seemed to be higher compared to the number

obtained in LR participants. This would suggest that the endothelium of the VHR is more prone to inflammation and requires repair. These results need to be further explored in a larger number of VHR participants in order to assess whether the number of ECFC colonies are related to cardiovascular events.

***Figure 2:** ECFC isolated from patient VHR-030 after CD45 depletion by autoMACS (50 x magnification)*

***Table 1:** Colony forming assay results of VHR and LR participants*

Cohort	**CPT® (Cell preparation tubes BD)**	**Ficoll separation**	**AutoMACS depletion of CD45+ fraction**
VHR *(% of colonies)*	(n=4) 3.12%	(n=5) 1.24%	(n=5) 4.58%
LR *(% of colonies)*		(n=3) 0.346%	

LR: low risk; VHR: very high risk

III. Tables

<u>Table 1:</u> Proteins which levels were statistically different between the compared cohorts (ranked according to the difference in average NPX means).

VHR/MR Proteins *(n=49)*	VHR *(average)*	MR *(average)*	VHR-MR *(average difference)*	Absolute value VHR - MR *(average difference)*	p value	**VHR/LR Proteins *(n=52)***	VHR *(average)*	LR *(average)*	VHR-LR *(average difference)*	Absolute value VHR - LR *(average difference)*	p value	**MR/LR Proteins *(n=8)***	MR *(average)*	LR *(average)*	MR-LR *(average difference)*	p value	**VHR/non-VHR Proteins *(n=72)***	VHR *(average)*	non-VHR *(average)*	VHR - non-VHR *(average difference)*	Absolute value VHR – non-VHR *(average difference)*	p value
REN	7.88	6.90	0.98	0.98	.001	**IL_6**	3.65	2.45	1.19	1.19	.043	**PAPPA**	2.30	1.99	0.31	.014	**REN**	7.88	6.87	1.01	1.01	.001
CHIT1	5.45	4.67	0.78	0.78	.043	**MMP_7**	9.57	8.50	1.06	1.06	.000	**CTSD**	4.59	4.29	0.30	.043	**GDF_15**	4.78	3.87	0.90	0.90	.002
FGF_21	6.55	5.79	0.76	0.76	.005	**MMP_12**	7.82	6.79	1.03	1.03	.010	**MMP_9**	3.45	3.15	0.30	.046	**MMP_12**	7.82	6.95	0.87	0.87	.000
MMP_12	7.82	7.12	0.70	0.70	.001	**HAOX1**	4.31	3.44	0.87	0.87	.007	**CTSL1**	4.84	4.54	0.29	.062	**MMP_7**	9.57	8.72	0.85	0.85	.000
GDF_15	4.78	4.10	0.67	0.67	.006	**ACE2**	4.10	3.33	0.77	0.77	.011	**TNFRSF11A**	4.73	4.46	0.27	.010	**CHIT1**	5.45	4.63	0.82	0.82	.054
MMP_7	9.57	8.93	0.64	0.64	.000	**TRAIL_R2**	5.53	4.80	0.73	0.73	.053	**LOX_1**	5.96	5.70	0.27	.024	**FGF_21**	6.55	5.79	0.77	0.77	.004
TRAIL_R2	5.53	5.01	0.53	0.53	.000	**PSP_D**	1.69	1.01	0.68	0.68	.038	**HO_1**	12.77	12.52	0.25	.055	**TIM**	7.39	6.68	0.71	0.71	.067
PSP_D	1.69	1.18	0.51	0.51	.004	**CEACAM8**	3.72	3.07	0.65	0.65	.000	**IL2_RA**	3.32	3.16	0.15	.067	**TRAIL_R2**	5.53	4.90	0.63	0.63	.000
PTX3	3.27	2.80	0.47	0.47	.000	**MMP_9**	3.78	3.15	0.63	0.63	.000						**ACE2**	4.10	3.50	0.61	0.61	.000

CEACAM8	3.72	3.25	0.47	0.47	.001	**PON3**	5.04	5.66	-0.61	0.61	.035						**PSP_D**	1.69	1.10	0.60	0.60	.003
OPN	4.12	3.66	0.46	0.46	.021	**PTX3**	3.27	2.74	0.53	0.53	.062						**CEACAM8**	3.72	3.16	0.56	0.56	.000
ACE2	4.10	3.66	0.44	0.44	.007	**LOX_1**	6.20	5.70	0.51	0.51	.000						**t_PA**	5.66	5.14	0.53	0.53	.067
TFF3	5.13	4.72	0.41	0.41	.019	**U_PAR**	4.47	3.98	0.49	0.49	.006						**OPN**	4.12	3.59	0.53	0.53	.015
TNF_R1	5.79	5.41	0.38	0.38	.032	**AZU1**	3.07	2.58	0.49	0.49	.000						**HAOX1**	4.31	3.79	0.52	0.52	.008
vWF	5.34	4.96	0.38	0.38	.062	**GLO1**	6.55	6.07	0.48	0.48	.000						**PTX3**	3.27	2.77	0.50	0.50	.000
AZU1	3.07	2.71	0.36	0.36	.004	**MMP_3**	5.60	5.11	0.48	0.48	.002						**PON3**	5.04	5.53	-0.49	0.49	.011
PON3	5.04	5.40	-0.36	0.36	.050	**TR_AP**	4.47	4.03	0.44	0.44	.019						**MMP_9**	3.78	3.30	0.48	0.48	.000
U_PAR	4.47	4.11	0.36	0.36	.001	**CTSD**	4.72	4.29	0.43	0.43	.003						**vWF**	5.34	4.87	0.47	0.47	.005
TR_AP	4.47	4.13	0.34	0.34	.000	**Gal_9**	7.80	7.39	0.41	0.41	.060						**CSTB**	5.07	4.60	0.47	0.47	.034
MMP_9	3.78	3.45	0.34	0.34	.040	**CD163**	6.16	5.75	0.41	0.41	.010						**FABP4**	5.07	4.60	0.46	0.46	.006
IL_18BP	4.89	4.56	0.33	0.33	.008	**MCP_1**	2.63	2.23	0.40	0.40	.012						**TNF_R1**	5.79	5.34	0.45	0.45	.050
FABP4	5.07	4.75	0.31	0.31	.055	**IL_1ra**	6.92	6.56	0.36	0.36	.010						**U_PAR**	4.47	4.05	0.42	0.42	.000
IL2_RA	3.62	3.32	0.30	0.30	.023	**CD4**	2.92	2.58	0.34	0.34	.017						**AZU1**	3.07	2.65	0.42	0.42	.000
SRC	7.02	7.32	-0.30	0.30	.061	**TNFRSF10A**	2.39	2.04	0.34	0.34	.009						**IL_18BP**	4.89	4.47	0.42	0.42	.003
PlGF	7.76	7.46	0.30	0.30	.017	**IL16**	5.58	5.24	0.34	0.34	.043						**TNFRSF11A**	5.01	4.59	0.41	0.41	.023
LTBR	2.62	2.33	0.29	0.29	.022	**DCN**	5.29	4.97	0.32	0.32	.034						**PlGF**	7.76	7.35	0.41	0.41	.003
Gal_4	2.91	2.62	0.29	0.29	.040	**JAM_A**	4.86	4.54	0.32	0.32	.021						**TR_AP**	4.47	4.08	0.39	0.39	.000
RETN	5.93	5.64	0.29	0.29	.033	**OPG**	2.64	2.35	0.29	0.29	.068						**Gal_4**	2.91	2.52	0.39	0.39	.032

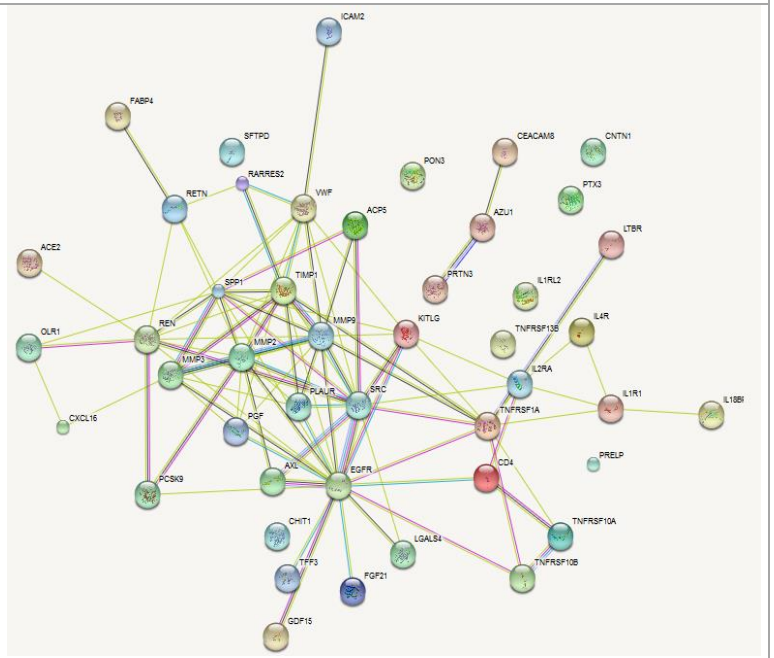
LOX_1	6.20	5.96	0.24	0.24	.036	**IDUA**	6.06	5.77	0.29	0.29	.016						**IL2_RA**	3.62	3.24	0.38	0.38	.005
CD4	2.92	2.68	0.24	0.24	.015	**Gal_3**	5.00	4.72	0.28	0.28	.006						**LOX_1**	6.20	5.83	0.37	0.37	.000
TNFRSF10A	2.39	2.16	0.23	0.23	.002	**CXCL1**	7.95	7.68	0.26	0.26	.049						**CCL3**	3.66	3.30	0.36	0.36	.062
IL_1RT1	5.55	5.33	0.22	0.22	.015	**LPL**	9.41	9.66	-0.25	0.25	.010						**DECR1**	5.90	5.55	0.35	0.35	.037
hOSCAR	10.44	10.22	0.21	0.21	.000	**IL_4RA**	1.87	1.62	0.25	0.25	.018						**MMP_3**	5.60	5.26	0.34	0.34	.033
PRTN3	5.04	4.83	0.21	0.21	.020	**hOSCAR**	10.44	10.20	0.23	0.23	.001						**GLO1**	6.55	6.23	0.33	0.33	.000
ICAM_2	4.03	3.82	0.20	0.20	.008	**CNTN1**	1.72	1.94	-0.22	0.22	.001						**CTSZ**	4.74	4.43	0.31	0.31	.041
CNTN1	1.72	1.92	-0.20	0.20	.004	**TNFRSF10C**	4.97	4.75	0.22	0.22	.003						**CD4**	2.92	2.63	0.29	0.29	.001
TNFRSF13B	7.63	7.44	0.19	0.19	.033	**PRTN3**	5.04	4.84	0.21	0.21	.010						**TNFRSF10A**	2.39	2.10	0.29	0.29	.000
EGFR	2.16	2.34	-0.18	0.18	.025	**LDL_receptor**	2.85	2.65	0.20	0.20	.005						**CD163**	6.16	5.88	0.28	0.28	.015
IL_4RA	1.87	1.69	0.18	0.18	.007	**IGFBP_1**	2.97	3.17	-0.20	0.20	.037						**CTSD**	4.72	4.44	0.28	0.28	.005
SPON1	0.64	0.46	0.17	0.17	.011	**MARCO**	5.05	4.89	0.16	0.16	.010						**SRC**	7.02	7.30	-0.28	0.28	.036
SCF	8.61	8.77	-0.16	0.16	.015	**PRELP**	6.33	6.18	0.15	0.15	.024						**RETN**	5.93	5.65	0.28	0.28	.052
RARRES2	10.54	10.41	0.13	0.13	.030	**LEP**	5.18	5.32	-0.14	0.14	.049						**MCP_1**	2.63	2.37	0.27	0.27	.016
CXCL16	4.54	4.41	0.13	0.13	.052	**IL_17RA**	2.32	2.19	0.13	0.13	.027						**Gal_9**	7.80	7.54	0.26	0.26	.041
IL1RL2	3.15	3.03	0.12	0.12	.039	**Notch_3**	2.45	2.34	0.12	0.12	.001						**IL_1ra**	6.92	6.66	0.25	0.25	.009

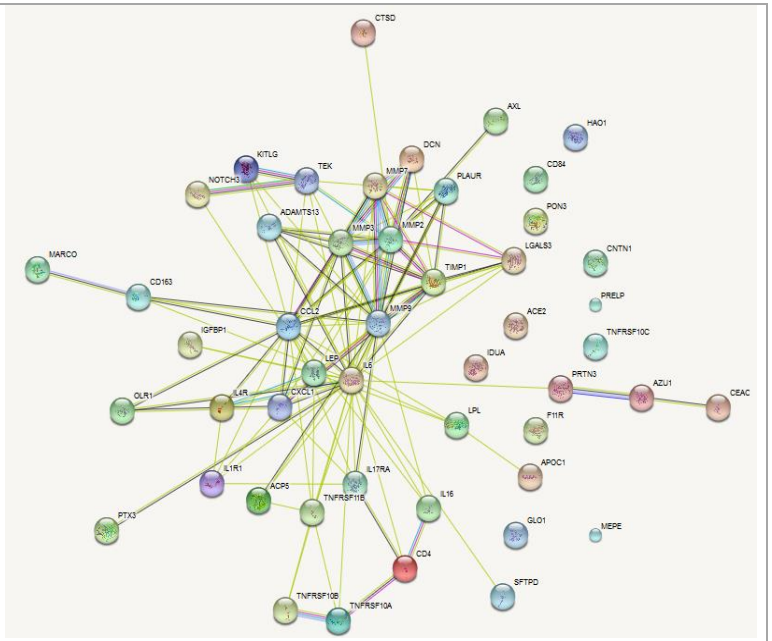
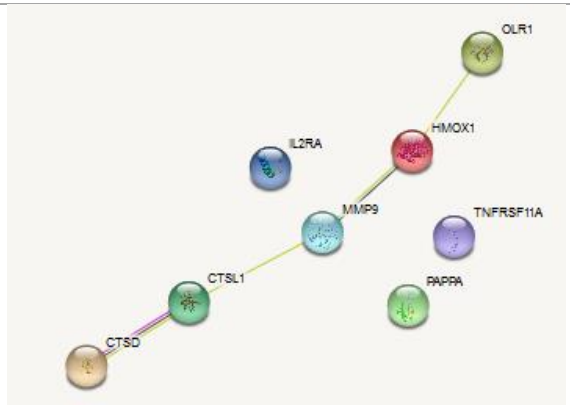
AXL	6.53	6.62	-0.09	0.09	.061	**CD84**	5.00	4.93	0.07	0.07	.068						**LPL**	9.41	9.64	-0.23	0.23	.007
PCSK9	0.92	0.82	0.09	0.09	.009	**MEPE**	2.06	2.12	-0.07	0.07	.001						**JAM_A**	4.86	4.63	0.23	0.23	.022
MMP_2	3.57	3.63	-0.06	0.06	.040	**AXL**	6.53	6.48	0.06	0.06	.051						**hOSCAR**	10.44	10.21	0.22	0.22	.000
PRELP	6.33	6.35	-0.02	0.02	.010	**TIE2**	7.05	7.00	0.04	0.04	.048						**IDUA**	6.06	5.84	0.21	0.21	.008
						MMP_2	3.57	3.61	-0.04	0.04	.000						**IL_4RA**	1.87	1.66	0.21	0.21	.002
						ADAM_TS13	5.33	5.31	0.02	0.02	.039						**CNTN1**	1.72	1.93	-0.21	0.21	.000
						SCF	8.61	8.61	0.00	0.00	.046						**SPON1**	0.64	0.43	0.21	0.21	.012
																	EGFR	2.16	2.36	-0.21	0.21	.012
																	PRTN3	5.04	4.83	0.21	0.21	.001
																	ICAM_2	4.03	3.85	0.18	0.18	.051
																	IGFBP_1	2.97	3.15	-0.18	0.18	.011
																	RARRES2	10.54	10.37	0.17	0.17	.012
																	Gal_3	5.00	4.83	0.17	0.17	.007
																	ITGB2	4.14	4.29	-0.15	0.15	.043
																	IL1RL2	3.15	3.00	0.15	0.15	.011
																	MPO	3.22	3.08	0.14	0.14	.057
																	PD_L2	2.57	2.45	0.12	0.12	.043
																	PCSK9	0.92	0.82	0.10	0.10	.003

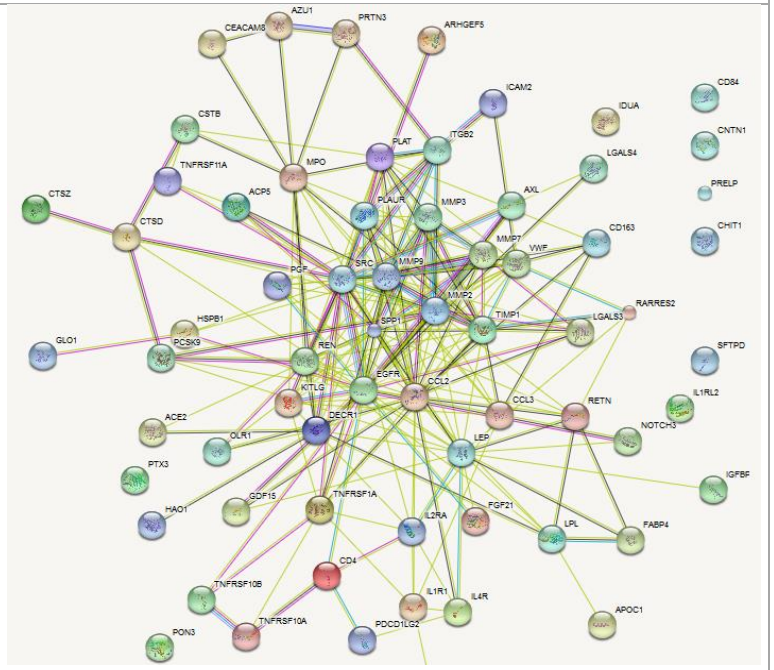
																	CD84	5.00	4.91	0.09	0.09	.028
																	SCF	8.61	8.69	-0.08	0.08	.009
																	Notch_3	2.45	2.37	0.08	0.08	.012
																	LDL_receptor	2.85	2.77	0.08	0.08	.008
																	LEP	5.18	5.25	-0.07	0.07	.055
																	PRELP	6.33	6.27	0.07	0.07	.001
																	HSP_27	10.20	10.26	-0.06	0.06	.028
																	MMP_2	3.57	3.62	-0.05	0.05	.000
																	AXL	6.53	6.55	-0.02	0.02	.030

Proteins were ranked according to the difference between the NPX means of the compared cohorts. LR: low risk; MR: moderate risk; non-VHR: non-very high risk; NPX: normalised protein ratio; VHR: very high risk

<u>Table 2:</u> Biological processes, pathways and disease implications shared by the proteins that differentiated between two CVD risk groups.

Proteins	*Protein number*	Gene Ontology Biological processes 2017	Reactome pathway 2016	Jensen DISEASES	STRING (version 10.5)
VHR/ MR proteins	49	1.Positive regulation of cell proliferation 2.Neutrophil degranulation 3.Regulation of stem cell proliferation 4.Regulation of osteoblast proliferation 5.Regulation of cell proliferation involved in compound eye morphogenesis 6.Regulation of leukocyte proliferation 7.Regulation of cell proliferation in bone marrow 8.Regulation of cell proliferation involved in imaginal disc derived wing morphogenesis 9.Regulation of synoviocyte proliferation 10. Regulation of germ cell proliferation	1. Cytokine signalling in immune system 2. Signalling by interleukins 3.GRB2: SOS provides linkage to MAPK signaling for integrins 4.p130Cas linkage to MAPK signalling for integrins 5.GP1b.IX-V activation signalling 6. Integrin alphaIIb beta3 signalling 7.MAP2K and MAPK activation 8. Metabolism of angiotensinogen to angiotensins 9.VEGFA-VEGFR2 pathway 10. L1CAM interactions	1. Coronary artery disease 2. Cerebrovascular disease 3. Hypertension 4. Vasculitis 5. Endocarditis 6. Axanthem 7. Factor XI deficiency 8. Hyperglycaemia 9. Lung disease 10. Purpura	

VHR/LR proteins	52	1. Regulation of store-operated calcium entry 2. Neutrophil degranulation 3. Acute inflammatory response 4. Inflammatory response 5. Inflammatory response to wounding 6. Chronic inflammatory response 7. Cellular response to lipopolysaccharide 8. Membrane fusion involved in viral entry into host cell 9. Viral entry into host cell via pilus retraction 10. Viral entry into host cell	1. TP53 regulates transcription of death receptors and ligands 2. CS.DS degradation 3. Peptide hormone metabolism 4. Metabolism of angiotensinogen to angiotensins 5. Diseases associated with glycosaminoglycan metabolism 6. Glycosaminoglycan metabolism 7.Chondroitin sulfate/dermatan sulfate metabolism 8. TP53 regulates transcription of cell death genes 9. Heparan sulfate/heparin (HS-GAG) metabolism 10. Transcriptional regulation of white adipocyte differentiation	1. Coronary artery disease 2. Endocarditis 3. Arthritis 4. Synovitis 5. Vasculitis 6. Hypertension 7. Tetanus neopnatorum 8. Hyperglycaemia 9. Rabies 10. Mumps	
MR/LR proteins	8	1. Cellular protein catabolic process 2. Osteoclast differentiation 3. Multinuclear osteoclast differentiation 4. Osteoclast development 5. Positive regulation of fever generation by positive regulation of prostaglandin secretion 6. response to granulocyte macrophage colony stimulating factor 7. Response to interleukin-21	1. TNF receptor superfamily (TNFSF) members mediating non-canonical NF-kB pathway 2. Regulation of insulin like growth factor (IGF) transport and uptake by insulin like growth factor binding protein 3. Metabolism of angiotensinogen to angiotensins 4. Collagen degradation 5. TNFR2 non-canonical NF-kB pathway	1. Osteoporosis 2. Cysticercosis 3. Tricuspid valve insuffiency 4. Neuronal ceroid lipofuscinosis 5. Paragonimiasis 6. Placental abruption 7. Inclusion cell disease 8. Placental insufficiency 9. Edwards syndrome	

		8. Response to erythropoietin 9. Response to interleukin- 13 10. Response to interleukin 17	6. Peptide hormone metabolism 7. Degradation of the extracellular matrix 8. MHC class II antigen presentation 9. Metabolism of proteins 10. Extracellular matrix organisation	10. Fascioliasis	
VHR/ non-VHR proteins	72	1. Neutrophil degranulation 2. Positive regulation of protein kinase B signalling 3. Negative regulation of apoptotic process 4. Acute inflammatory response 5. Inflammatory response to wounding 6. Chronic inflammatory response 7. Inflammatory response 8. Osteoclast development 9. Negative regulation of neuron apoptotic process 10. Positive regulation of peptidyl-tyrosine phosphorylation	1. Metabolism of angiotensinogen to angiotensins 2. peptide hormone metabolism 3. Cell surface interactions at the vascular wall 4. Signalling by interleukins 5. GRB2: SOS provides linkage to MAPK signalling for intergrins 6. p130Cas linkage to MAPK signalling for integrins 7. GP1b-IX-V activation signalling 8. Hemostatis signalling in immune system 9. Cytokine signalling in immune system 10. Transcriptional regulation of white adipocyte differentiation	1. Coronary artery disease 2. Arthritis 3. Hypertension 4. Cerebrovascular disease 5. Hyperglycaemia 6. Vasculitis 7. Lung disease 8. Purpura 9. Endocarditis 10. Synovitis	

This table represents the Gene Ontology Biological processes 2017, the Reactome pathway 2016 and the Jensesn diseases pathways that are relative to each set of proteins that were able to differentiate between each compared risk groups. The biological pathways are ranked according to their p value and provided by the Enrichr database (http://amp.pharm.mssm.edu/Enrichr/). In the STRING generated pathways, the network nodes represent proteins and

the coloured nodes represent a first shell of interaction whereas white nodes represent a second shell of interaction. Empty nodes are proteins of unknown 3D structure whereas filled nodes are known or predicted 3D structures. Blue lines represent a known interaction from curated databases whereas a purple line represents an experimentally determined known interaction. Green lines represent predicted interactions based on gene neighbourhood, red lines represent predicted interactions based on gene fusions and blue lines represent predicted interactions based o gene co-occurrence. Light green lines represent textmining, black lines represent co-expression and light blue lines represent protein homology. Szklarczyk et al. Nucleic Acids Res. 2015 43(Database issue):D447-52. *LR: low risk; MR: moderate risk; non-VHR: non-very high risk VHR: very high risk.*

***Table 3:* An example of the inter-correlation (Pearson correlation) analysis within measured proteins**

		TR_AP	MMP_7	PTX3	HOSCAR	TRAIL_R2	CEACAM8	U_PAR	MMP_12	REN	TNFRSF10A	IL_4RA	FGF_21	GDF_15	AZU1	ACE2	PSP_D	IL_18BP	CNTN1	PCSK9	PIgR	NT_pro_BNP	SPON1
TR_AP	Pearson Correlation	1	.384**	.174**	.202**	.356**	.259**	.388**	.321**	.274**	.265**	.143*	.289**	.444**	.280**	.381**	.216**	.406**	-.104	.284**	.142*	.198**	.360**
	Sig. (2-tailed)		.000	.003	.000	.000	.000	.000	.000	.000	.000	.014	.000	.000	.000	.000	.000	.000	.072	.000	.015	.001	.000
MMP_7	Pearson Correlation	.384**	1	.240**	.444**	.636**	.366**	.562**	.589**	.454**	.544**	.464**	.360**	.686**	.279**	.413**	.377**	.553**	-.171**	.173**	.445**	.447**	.534**
	Sig. (2-tailed)	.000		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.003	.003	.000	.000	.000
PTX3	Pearson Correlation	.174**	.240**	1	.398**	.419**	.508**	.400**	.260**	.254**	.347**	.361**	.161**	.413**	.288**	.275**	.044	.414**	-.258**	.059	.254**	.449**	.279**
	Sig. (2-tailed)	.003	.000		.000	.000	.000	.000	.000	.000	.000	.000	.005	.000	.000	.000	.451	.000	.000	.311	.000	.000	.000
HOSCAR	Pearson Correlation	.202**	.444**	.398**	1	.577**	.457**	.564**	.437**	.331**	.451**	.433**	.237**	.439**	.158**	.291**	.318**	.575**	-.168**	.119*	.370**	.315**	.353**
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.000	.000	.000	.000	.000	.000	.000	.006	.000	.000	.000	.004	.040	.000	.000	.000
TRAIL_R2	Pearson Correlation	.356**	.636**	.419**	.577**	1	.541**	.806**	.504**	.471**	.766**	.583**	.477**	.818**	.243**	.409**	.320**	.695**	-.275**	.181**	.440**	.646**	.602**
	Sig. (2-tailed)	.000	.000	.000	.000		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.002	.000	.000	.000
CEACAM8	Pearson Correlation	.259**	.366**	.508**	.457**	.541**	1	.583**	.244**	.329**	.421**	.375**	.286**	.404**	.608**	.277**	.086	.450**	-.352**	.101	.255**	.413**	.259**
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.000	.000	.000	.000	.000	.000	.000	.000	.000	.139	.000	.000	.082	.000	.000	.000
U_PAR	Pearson Correlation	.388**	.562**	.400**	.564**	.806**	.583**	1	.476**	.387**	.658**	.521**	.438**	.765**	.375**	.287**	.327**	.755**	-.185**	.284**	.351**	.663**	.620**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.001	.000	.000	.000	.000
MMP_12	Pearson Correlation	.321**	.589**	.260**	.437**	.504**	.244**	.476**	1	.398**	.420**	.364**	.196**	.530**	.121*	.330**	.450**	.494**	-.078	.170**	.462**	.325**	.439**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000		.000	.000	.000	.001	.000	.037	.000	.000	.000	.180	.003	.000	.000	.000
REN	Pearson Correlation	.274**	.454**	.254**	.331**	.471**	.329**	.387**	.398**	1	.321**	.250**	.164**	.465**	.208**	.353**	.255**	.377**	-.240**	.068	.258**	.256**	.283**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000		.000	.000	.005	.000	.000	.000	.000	.000	.000	.244	.000	.000	.000
TNFRSF10A	Pearson Correlation	.265**	.544**	.347**	.451**	.766**	.421**	.658**	.420**	.321**	1	.573**	.469**	.630**	.176**	.384**	.279**	.596**	-.175**	.317**	.379**	.511**	.496**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.000		.000	.000	.000	.002	.000	.000	.000	.003	.000	.000	.000	.000
IL_4RA	Pearson Correlation	.143*	.464**	.361**	.433**	.583**	.375**	.521**	.364**	.250**	.573**	1	.317**	.528**	.069	.469**	.189**	.474**	-.160**	.131*	.381**	.473**	.439**
	Sig. (2-tailed)	.014	.000	.000	.000	.000	.000	.000	.000	.000	.000		.000	.000	.234	.000	.001	.000	.006	.024	.000	.000	.000
FGF_21	Pearson Correlation	.289**	.360**	.161**	.237**	.477**	.286**	.438**	.196**	.164**	.469**	.317**	1	.479**	.178**	.245**	.053	.311**	-.229**	.187**	.109	.370**	.292**
	Sig. (2-tailed)	.000	.000	.005	.000	.000	.000	.000	.001	.005	.000	.000		.000	.002	.000	.363	.000	.000	.001	.062	.000	.000
GDF_15	Pearson Correlation	.444**	.686**	.413**	.439**	.818**	.404**	.765**	.530**	.465**	.630**	.528**	.479**	1	.237**	.440**	.339**	.710**	-.212**	.181**	.333**	.665**	.661**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000		.000	.000	.000	.000	.000	.002	.000	.000	.000
AZU1	Pearson Correlation	.280**	.279**	.288**	.158**	.243**	.608**	.375**	.121*	.208**	.176**	.069	.178**	.237**	1	.204**	.029	.285**	-.106	.133*	-.047	.185**	.171**
	Sig. (2-tailed)	.000	.000	.000	.006	.000	.000	.000	.037	.000	.002	.234	.002	.000		.000	.618	.000	.068	.022	.424	.001	.003
ACE2	Pearson Correlation	.381**	.413**	.275**	.291**	.409**	.277**	.287**	.330**	.353**	.384**	.469**	.245**	.440**	.204**	1	.206**	.316**	-.142*	.225**	.269**	.223**	.292**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000		.000	.000	.015	.000	.000	.000	.000
PSP_D	Pearson Correlation	.216**	.377**	.044	.318**	.320**	.086	.327**	.450**	.255**	.279**	.189**	.053	.339**	.029	.206**	1	.297**	-.090	.190**	.412**	.189**	.310**
	Sig. (2-tailed)	.000	.000	.451	.000	.000	.139	.000	.000	.000	.000	.001	.363	.000	.618	.000		.000	.123	.001	.000	.001	.000
IL_18BP	Pearson Correlation	.406**	.553**	.414**	.575**	.695**	.450**	.755**	.494**	.377**	.596**	.474**	.311**	.710**	.285**	.316**	.297**	1	-.058	.313**	.313**	.524**	.522**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000		.320	.000	.000	.000	.000
CNTN1	Pearson Correlation	-.104	-.171**	-.258**	-.168**	-.275**	-.352**	-.185**	-.078	-.240**	-.175**	-.160**	-.229**	-.212**	-.106	-.142*	-.090	-.058	1	.172**	-.105	-.244**	.084
	Sig. (2-tailed)	.072	.003	.000	.004	.000	.000	.001	.180	.000	.003	.006	.000	.000	.068	.015	.123	.320		.003	.071	.000	.151
PCSK9	Pearson Correlation	.284**	.173**	.059	.119*	.181**	.101	.284**	.170**	.068	.317**	.131*	.187**	.181**	.133*	.225**	.190**	.313**	.172**	1	.143*	.160**	.295**
	Sig. (2-tailed)	.000	.003	.311	.040	.002	.082	.000	.003	.244	.000	.024	.001	.002	.022	.000	.001	.000	.003		.014	.006	.000
PIgR	Pearson Correlation	.142*	.445**	.254**	.370**	.440**	.255**	.351**	.462**	.258**	.379**	.381**	.109	.333**	-.047	.269**	.412**	.313**	-.105	.143*	1	.242**	.290**
	Sig. (2-tailed)	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.062	.000	.424	.000	.000	.000	.071	.014		.000	.000

Highlighted cells show a significant correlation

***Table 4:* Significant proteins specific to the category of VHR participants within each group.**

Group 1: Primary and secondary ACS events								Group 2: Participants with previous MI, PCI or CABG						Group 3		Group 4	
ACS/Elective participants	p value	**ACS first events**	p value	**ACS recurrent MACE**	p value	***Specific to first CV events***	***Specific to recurrent MACE***	**Specific to participants with previous MI**	p value	**Specific to participants with previous PCI**	p value	**Specific to participants with previous CABG**	p value	**Diagnosis upon admission**	p value	**Follow-up MACE**	p value
Protein_BOC	<0.001	ADAM_TS13	<0.001	TNFSF13B	.002	*HOSCAR*	*TNFSF13B*	MMP_2	<0.001	CHIT1	.002	DCN	<0.001	OPN	<0.001	CCL16	.001
OPN	<0.001	OPN	<0.001	Protein_BOC	.004	*PCSK9*	*IL_18BP*	IGFBP_1	<0.001	SOD2	.013	IGFBP_2	<0.001	IL_6	<0.001	DCN	.001
VEGF_D	<0.001	VEGF_D	<0.001	IL_18BP	.004	*CHI3L1*	*ADM*	SCF	<0.001	PRSS27	.024	EGFR	<0.001	NT_pro_BNP	<0.001	BNP	.001
ADAM_TS13	<0.001	Protein_BOC	<0.001	ADM	.005	*HBEGF*	*TNFRSF11A*	CD93	.001	NEMO	.036	OPG	<0.001	OPG	<0.001	RARRES2	.001
PIgR	<0.001	PD_L2	<0.001	TNFRSF11A	.008	*APN*	*TNF_R2*	PAI	.003	HB_EGF	.037	SPON1	<0.001	MB	<0.001	AMBP	.003
TFPI	<0.001	TR_AP	<0.001	TNF_R2	.009	*MMP_9*	*IL2_RA*	ALCAM	.004	BMP_6	.057	TIMP4	<0.001	Protein_BOC	<0.001	NT_pro_BNP	.003
IL_6	<0.001	PIgR	<0.001	CCL3	.013	*HAOX1*	*IL_4RA*	AXL	.004	Dkk_1	.058	NT_pro_BNP	<0.001	MARCO	<0.001	PAPPA	.004
MB	<0.001	AZU1	<0.001	IL2_RA	.013	*U_PAR*	*VSIG2*	TNFSF13B	.006	CD84	.065	TF	<0.001	CTRC	<0.001	TFF3	.004
LPL	<0.001	CCL15	<0.001	IL_4RA	.013	*IL_1ra*	*IGFBP_7*	PARP_1	.006	CEACAM8	.066	BNP	<0.001	PD_L2	<0.001	IL_1RT1	.005
CCL15	<0.001	HOSCAR	<0.001	CNTN1	.014		*EGFR*	SLAMF7	.007			IL27	<0.001	VEGF_D	<0.001	TNF_R1	.005
PSGL_1	<0.001	LPL	<0.001	RAGE	.019		*PAR_1*	TGM2	.016			TNF_R1	<0.001	AZU1	<0.001	ADM	.006
hOSCAR	<0.001	RARRES2	<0.001	CTSL1	.020		*CHIT1*	AP_N	.017			LTBR	<0.001	CCL15	<0.001	IL_6	.011
MARCO	<0.001	CCL3	0.001	FGF_23	.021		*CD163*	GRN	.018			TIM	<0.001	BNP	<0.001	PD_L2	.014

CDH5	<0.001	PSP_D	0.001	SLAMF7	.023		*SPON2*	XCL1	.020			MMP_7	<0.001	CDH5	<0.001	PTX3	.015
IL16	<0.001	CDH5	0.001	VSIG2	.024		*FAS*	IL_17RA	.026			MB	<0.001	TNF_R1	<0.001	TRAIL_R2	.016
PSP_D	<0.001	PSGL_1	0.001	hOSCAR	.024		*TLT_2*	VEGF_D	.030			IGFBP_7	<0.001	PIgR	<0.001	MMP_3	.017
OPG	<0.001	PI3	0.001	TNFRSF14	.027			PSGL_1	.038			OPN	<0.001	LPL	<0.001	PRTN3	.021
PD_L2	<0.001	AGRP	0.001	GDF_15	.029			CPB1	.040			MMP_3	<0.001	DCN	.001	REN	.023
IL_17D	<0.001	AGRP	0.002	THBS2	.031			STK4	.043			HAOX1	<0.001	GDF_15	.001	THBS2	.026
HB_EGF	<0.001	IL_6	0.002	IGFBP_7	.033			IDUA	.048			RETN	<0.001	IL_17D	.001	Gal_4	.027
TGM2	<0.001	PRELP	0.002	IL16	.033			CPA1	.055			LDL_receptor	<0.001	PRTN3	.002	TNFRSF14	.028
PRSS8	<0.001	CTSL1	0.003	TFF3	.036			HO_1	.067			CTSZ	<0.001	CHI3L1	.002	LTBR	.028
TNF_R1	<0.001	CXCL16	0.003	MMP_2	.038			IL1RL2	.068			TM	<0.001	FGF_21	.002	Gal_3	.030
AMBP	<0.001	MB	0.003	PLC	.042			TFPI	.068			CCL15	.001	AMBP	.002	IDUA	.030
FABP2	<0.001	OPG	0.004	EGFR	.042							FAS	.001	RETN	.002	GDF_15	.035
PI3	<0.001	TNFRSF13B	0.004	PAR_1	.043							PTX3	.002	TNFRSF10C	.002	FGF_23	.035
TNFRSF13B	<0.001	TFPI	0.004	CHIT1	.043							GLO1	.002	TR_AP	.003	EPHB4	.035
CTRC	<0.001	CD93	0.004	TNF_R1	.047							PGLYRP1	.002	ST2	.003	IL2_RA	.036
CCL3	.001	BMP_6	0.006	PRSS8	.049							FABP4	.002	BMP_6	.003	SHPS_1	.039
CTSL1	.001	RAGE	0.007	TM	.055							TNFRSF10C	.003	CCL16	.004	KLK6	.045
PRELP	.001	TM	0.010	CD163	.055							ICAM_2	.004	PSP_D	.004	PI3	.046
ST2	.001	MPO	0.011	MMP_12	.056							PI3	.008	PTX3	.004	IL_4RA	.047

Gal_9	.001	ST2	0.011	TF	.058							ST2	.011	t_PA	.005	VSIG2	.051
t_PA	.001	Notch_3	0.012	SPON2	.059							IL_6	.013	SRC	.007	PIgR	.053
AGRP	.001	IL16	0.013	CDH5	.063							t_PA	.019	FABP2	.009	PLC	.053
DCN	.002	AMBP	0.015	FAS	.064							PRTN3	.033	G_T	.009	U_PAR	.055
GDF_15	.002	PCSK9	0.015	LTBR	.064							AGRP	.035	MMP_3	.009	IL1RL2	.061
TR_AP	.002	MARCO	0.016	PlGF	.065							FGF_21	.044	PGLYRP1	.010	PAR_1	.069
TIE2	.003	TF	0.017	PD_L2	.066							HSP_27	.047	PRSS8	.010	CXCL16	.005
BMP_6	.003	TNF_R1	0.018	TLT_2	.069							CCL24	.050	IL_1ra	.011	TR	.014
RARRES2	.003	CTSD	0.018									RARRES2	.051	TIE2	.013	ADAM_TS13	.016
CCL17	.004	PRTN3	0.020									MEPE	.055	CTSZ	.013	IL_1RT2	.025
AZU1	.005	MMP_12	0.020									PSP_D	.056	U_PAR	.014	CCL22	.034
RETN	.006	GDF_15	0.024									GIF	.062	MPO	.017	AGRP	.043
CTSZ	.008	FABP2	0.024											RARRES2	.018	CD93	.060
CD93	.010	SERPINA12	0.025											CCL17	.019		
XCL1	.011	CTRC	0.025											PlGF	.019		
CXCL16	.012	CHI3L1	0.028											TFF3	.020		
GRN	.013	HBEGF	0.029											TIMP4	.021		
PRTN3	.017	APN	0.029											TNFRSF14	.026		
EPHB4	.017	PGLYRP1	0.029											IL_4RA	.029		
CTSD	.018	SLAMF7	0.029											PI3	.030		
TFF3	.018	RETN	0.033											PON3	.031		
GLO1	.021	CNTN1	0.035											SPON1	.031		
uPA	.021	AP_N	0.035											IL16	.032		

NT_pro_BNP	.024	GLO1	0.036											EPHB4	.036		
TNFRSF14	.025	t_PA	0.036											TNFRSF13B	.038		
SCF	.026	NT_pro_BNP	0.038											TM	.039		
MPO	.026	HB_EGF	0.038											TF	.040		
IL_1RT1	.028	MMP_9	0.040											GDF_2	.040		
Notch_3	.029	DCN	0.042											LTBR	.040		
GIF	.029	IL_17D	0.043											hOSCAR	.042		
COL1A1	.030	GRN	0.044											Gal_9	.046		
LTBR	.034	COL1A1	0.045											PRELP	.048		
TM	.035	CTSZ	0.046											COL1A1	.052		
SOD2	.037	TIE2	0.049											PAR_1	.060		
MMP_12	.037	HAOX1	0.050											ADAM_TS13	<0.001		
BNP	.038	TGM2	0.050											HB_EGF	.001		
PGLYRP1	.040	Gal_9	0.051											TFPI	.001		
ANG_1	.040	TGM2	0.052											XCL1	.005		
PLC	.043	THBS2	0.057											Ep_CAM	.006		
THPO	.046	MMP_2	0.057											PSGL_1	.008		
GDF_2	.046	FGF_23	0.057											CD93	.010		
TIMP4	.048	U_PAR	0.059											CXCL16	.011		
Dkk_1	.049	IL_1ra	0.060											CD84	.013		
AP_N	.049	XCL1	0.067											AGRP	.027		
RAGE	.050	SCF	0.070											IL_17RA	.029		
PlGF	.051													Dkk_1	.030		

PDGF_subunit_B	.057													PDGF_subunit_B	.032		
THBS2	.060													TGM2	.046		
G_T	.063													SCF	.054		
PRSS27	.063													MMP_2	.061		
IGFBP_2	.064													RAGE	.069		
SERPINA12	.065																
PTX3	.066																
Group 5: Medical Therapy										**Group 6: Angiogram outcome**							
Statin therapy	p value	**Anti-platelet therapy**	p value	**Anti-hypertensive therapy**	p value	**Anti-anginal therapy**	p value	**Diuretic therapy**	p value	**Plaque burden**	p value						
IDUA	.003	IGFBP_2	<0.001	Ep_CAM	.002	PDGF_subunit_B	.001	MMP_2	<0.001	HAOX1	.001						
COL1A1	.020	OPG	<0.001	CCL24	.049	SOD2	.004	TR	.001	GIF	.008						
JAM_A	.020	CHI3L1	<0.001			ITGB1BP2	.009	FGF_21	.003	LEP	.009						
PAR_1	.066	PlGF	<0.001			STK4	.017	CD93	.004	Log_IL1RT2	.019						
		LDL_receptor	<0.001			DECR1	.020	vWF	.007	Log_CCL22	.020						
		IL2_RA	<0.001			SLAMF7	.032	PRTN3	.025	IL_4RA	.020						
		FAS	.001			XCL1	.036	GH	.032	THBS2	.022						
		GLO1	.003			TGM2	.049	PON3	.044	Log_PARP1	.024						
		TNFRSF10C	.004			PARP_1	.054	CTSD	.063	ACE2	.034						
		ST2	.006					AZU1	.068	Log_APN	.037						

		t_PA	.018							IL1RL2	.045						
		MEPE	.029							AMBP	.045						
		ADAM_TS13	.041							ITGB2	.047						
		AP_N	.065							CA_5A	.050						
										log_IL16	.050						
										CCL3	.052						
										SPON2	.052						
										ALCAM	.056						
										MERTK	.062						
										CD84	.063						
Group 7: Cardiovascular disease and comorbidities																	
Diabetes	p value	**Heart failure**	p value	**Hypertension**	p value	**Dyslipidaemia**	p value	**Arthritis**	p value	**Depression**	p value	**Cancer**	p value	**GFR<60**	p value		
GIF	.001	EGFR	.006	AP_N	.007	G_T	.013	PAPPA	.016	Ep_CAM	.013	THPO	.032	TLT_2	.000		
CTSD	.001	CNTN1	.013	NEMO	.026			MCP_1	.018	CD40_L	.051	MMP_9	.043	TFPI	.003		
PON3	.007	CCL24	.030	PSGL_1	.047			TNFSF13B	.022	vWF	.052	BLM_hydrolase	.049	CA5A	.035		
TIE2	.012			ITGB1BP2	.062			FABP2	.024	ANG_1	.064						
LDL_receptor	.018							CPA1	.031								
MARCO	.023							Protein_BOC	.038								
PECAM_1	.034							BMP_6	.048								
PSP_D	.037							SELE	.056								
IL_18	.066																

Proteins ranked according to the p value. CABG: Coronary artery bypass grafting; CV: Cardiovascular; GFR: Glomerular filtration rate in ml/min; MACE: major adverse cardiovascular events; MI: myocardial infarction; PCI: percutaneous coronary intervention.

<u>Table 5:</u> Levels of proteins associated with MACE in cluster 1 and cluster 2 subgroups within the VHR cohort.

Average of the proteins that were higher in Cluster 1					Average of the proteins that were higher in Cluster 2				
Protein	Cluster 1 (n=149)		Cluster 2 (n=59)		Protein	Cluster 1		Cluster 2	
	Mean	Std deviation	Mean	Std deviation		Mean	Std deviation	Mean	Std deviation
ADAM_TS13	5.355168	.1996098	5.252542	.2314690	PGLYRP1	6.721879	.4237260	7.233559	.5548711
ANG_1	8.541879	1.1283547	8.267458	.9941043	PRELP	6.256846	.2184128	6.517458	.2775794
CCL17	8.023356	1.0281449	7.970339	1.0271722	Protein BOC	4.088121	.3245504	4.213559	.3113196
CCL22	2.617763	1.1051860	2.546429	1.2423693	PRSS27	8.818523	.4414478	8.976271	.5430720
GLO1	6.609732	.6977405	6.410000	.6791678	PRSS8	9.541611	.3822446	9.858475	.3817736
IDUA	6.062752	.4735588	6.040169	.5031230	PRTN3	4.940987	0.4228972	5.314107	0.7100468
LDL receptor	2.905034	.5280326	2.705254	.6276650	PARP1	1.774803	0.7571636	1.851071	0.5703992
PDGF subunit B	9.494295	1.1110558	9.286949	1.0418787	PAPPA	2.302237	0.5138456	2.569643	1.0384568
PSGL1	4.521946	.2610418	4.458814	.2698409	PSP_D	1.618859	.7432566	1.876441	.6744596
SERPINA12	4.528456	1.0904715	4.430508	1.1329203	PTX3	3.158859	.4856293	3.560169	.4933191
CTRC	9.784765	.7089768	9.556271	1.0638024	RAGE	5.163154	.3790602	5.571864	.4983852
EGFR	2.197047	.1969823	2.051525	.2038070	RARRES2	10.455503	.2858237	10.754915	.3207070
GDF_2	3.507315	.5217158	3.370847	.5186132	REN	7.665906	.9468183	8.430339	1.0026911
TGM2	8.210987	0.6676426	7.840714	0.9638651	RETN	5.742829	0.4316355	6.426429	0.7207482
TIE2	7.048591	.2471459	7.042373	.3046555	COL1A1	2.835302	.3659171	3.097119	.5005516
SCF	8.632368	0.4681056	8.560714	0.6714621	CPB1	3.495235	.5806760	3.875593	.9335772
HBEGF	4.994605	0.7470240	4.864643	0.6468777	CSTB	4.869664	.5657755	5.587119	.6883264
Average of the proteins that were higher in Cluster 2					CTSD	4.658792	.3619745	4.883220	.4769857
Protein	Cluster 1		Cluster 2		CTSL1	4.881382	0.4138465	5.273750	0.4137701
	Mean	Std deviation	Mean	Std deviation	CTSZ	4.588054	.3830314	5.112373	.4131266
ACE2	4.026913	.6270328	4.286610	.8369271	CXCL16	4.433758	.2687018	4.810000	.3841246
ADM	6.838993	.3558047	7.643390	.4509515	DCN	5.166184	0.3057267	5.639107	0.5482046
AGRP	3.961711	0.3151532	4.398036	0.5890467	EPHB4	1.032500	0.2054530	1.491964	0.3178531
AMBP	5.969463	.1836705	6.185254	.2183587	FAS	3.998947	0.4333317	4.443929	0.4693852
APN	4.262632	0.2658380	4.396250	0.5151189	Dkk_1	9.186174	.7108128	9.318136	.5625633
AZU1	3.009408	0.6371379	3.233929	0.8160764	FABP2	8.562550	.9754979	8.753729	.8697560
BMP6	4.513947	0.4551779	4.671071	0.6070965	FGF_23	2.728322	.6505272	3.526271	1.3485981
BNP	2.379933	1.3444121	4.417288	2.0913631	GT	2.042483	.6847587	2.210000	.9489722
CCL15	5.803893	.4723113	6.464915	.6229058	Gal_3	4.957114	.3450329	5.113051	.3239799
CCL16	5.802081	.4779210	6.329661	.3981161	Gal_4	2.767987	.5927740	3.271017	.5694695
CCL3	3.513423	.4633876	4.040508	.5545655	Gal_9	7.681879	.3554277	8.108814	.3907335
CD163	6.072819	.4606768	6.374237	.5835805	GDF_15	4.439128	.4951657	5.623898	.6443629
CD93	8.374698	.3143581	8.885932	.3052366	SHPS_1	2.651745	.3502021	3.088814	.4220806
CDH5	1.990067	.2980488	2.168644	.2981174	SLAMF7	1.435000	0.3717170	1.663393	0.5681014
CEACAM8	3.577181	.5398562	4.068814	.7484791	SOD2	9.248523	.2511390	9.285763	.2608620
CHIT1	5.337315	1.2879974	5.732034	1.7317450	SPON2	10.040537	.1952953	10.318644	.1539084
CNTN1	1.705235	.2308433	1.749661	.3457125	ST2	3.138926	.6300130	3.752203	.9017934
GIF	6.483691	1.2045221	6.546610	1.2630022	t_PA	5.572886	.7192886	5.894576	.5872505
GRN	5.749732	.2876676	6.003559	.3237590	TF	5.524295	.2533779	5.895085	.3872020
HOSCAR	10.382483	.2335100	10.570847	.2062580	TFF3	4.842105	0.3516068	5.922857	0.7566739
IGFBP_2	6.698389	.6738765	7.740847	.6441602	TFPI	7.994698	.3658950	8.186441	.3521352
IGFBP_7	3.283154	.2979527	3.878475	.5175433	THPO	2.517651	.3643977	2.542881	.2968458
IL_18BP	4.698322	.3142395	5.377627	.4812424	TIMP4	3.536779	.4419678	4.167458	.6095351
IL_1RT1	5.451275	.2467198	5.791356	.3247251	TLT_2	3.884430	.4188101	4.249831	.4746632
IL_1RT2	4.311812	.3012004	4.388983	.4735074	TM	8.181275	.3051435	8.587458	.3654501
IL4RA	1.768618	0.2535184	2.142500	0.3932256	TNFR1	5.516776	0.2876454	6.539286	0.7024033
KLK6	5.884013	0.2966597	6.228929	0.4223470	TNFR2	3.815395	0.3069458	4.704464	0.5448333
IL17D	2.150987	0.2974812	2.398214	0.6137192	TNFRSF10A	2.249262	.2560962	2.733220	.4853276
LPL	9.351342	.5427752	9.543559	.5635900	TNFRSF11A	4.729195	.3239568	5.714746	.8160362

LTBR	2.430263	0.2840762	3.129643	0.5416136	TNFRSF13B	7.528389	.3672967	7.888814	.3728737
MARCO	5.033490	.2542874	5.096271	.2738103	TNFRSF14	3.458523	.2703561	4.271525	.6062912
MB	6.340592	0.7588107	7.088571	1.0745679	TNFSF13B	7.556447	0.3559474	7.831964	0.4529235
MCP1	2.549276	0.4454035	2.850714	0.4448283	VISG2	2.608816	0.5683704	3.368571	0.9106490
MEPE	1.985000	0.3295070	2.254643	0.6331910	TR	3.669396	.5695496	3.959492	.7039529
MMP_12	7.653289	.7574820	8.238136	.7491961	TR_AP	4.414430	.3341496	4.610339	.3934111
MMP_2	3.460537	.2909067	3.841017	.4264638	TRAIL_R2	5.293490	.3653486	6.144237	.5981241
MMP_3	5.412953	.6303876	6.055424	.8914930	U_PAR	4.278322	.2870903	4.954068	.4477612
MPO	3.190872	.3631114	3.284407	.4951463	uPA	4.026316	0.3766596	4.221250	0.4264955
Notch_3	2.316443	.3441619	2.796441	.4712740	VEGF_D	7.126913	.4907480	7.324915	.5534107
NT_proBNP	1.181645	0.8249314	3.020536	1.7459672	XCL1	4.648456	.6113042	5.039831	.5090542
OPG	2.493487	0.3483590	3.038393	0.4779037	PI3	4.550268	.5245796	5.296271	.8310004
OPN	3.874362	.5524140	4.724407	.6137288	PIgR	7.257114	.1622823	7.369153	.1513080
PAR_1	8.105503	.3933732	8.498475	.3130375	PLC	5.517987	.2911301	6.328983	.4699072
PD_L2	2.500000	.3130215	2.758475	.3962010	PlGF	7.585101	.2452429	8.188814	.5325899

<u>Table 6:</u> Correlation analysis between inflammatory biomarkers, age, BMI, systolic blood pressure, cholesterol levels and CRP levels

		Age	BMI	Systolic BP	Cholesterol (mmol/L)	IFN-γ plasma levels (pg/ml)	IL-10 plasma levels (pg/ml)	IL-12p70 plasma levels (pg/ml)	IL-13 plasma levels (pg/ml)	IL-1β plasma levels (pg/ml)	IL-2 plasma levels (pg/ml)	IL-4 plasma levels (pg/ml)	IL-6 plasma levels (pg/ml)	IL-8 plasma levels (pg/ml)	CRP levels (mg/dl)
Age	Pearson Correlation	1	.076	.214**	-.306**	.075	.124*	.151**	.105	.061	.196**	-.074	.456**	.368**	.101
	Sig. (2-tailed)		.172	.000	.000	.167	.022	.006	.117	.525	.001	.347	.000	.000	.084
	N	344	320	296	318	339	339	332	225	110	308	163	343	343	294
BMI	Pearson Correlation	.076	1	.145*	-.036	.018	.033	-.090	.034	.122	-.045	.039	.225**	.089	.073
	Sig. (2-tailed)	.172		.016	.540	.748	.563	.112	.629	.223	.447	.630	.000	.114	.229
	N	320	320	279	296	315	315	311	208	102	285	159	319	319	272
Systolic BP	Pearson Correlation	.214**	.145*	1	.072	.064	-.035	.077	.121	.157	.061	.007	.020	.122*	-.111
	Sig. (2-tailed)	.000	.016		.237	.276	.557	.198	.093	.120	.323	.934	.737	.037	.079
	N	296	279	296	275	291	291	285	193	99	266	141	295	295	251
Cholesterol (mmol/L)	Pearson Correlation	-.306**	-.036	.072	1	-.105	-.154**	-.089	-.140*	-.195*	-.127*	-.020	-.200**	-.234**	-.115
	Sig. (2-tailed)	.000	.540	.237		.064	.006	.120	.043	.050	.033	.807	.000	.000	.059
	N	318	296	275	318	314	313	307	209	102	283	151	317	317	271
IFN-γ plasma levels (pg/ml)	Pearson Correlation	.075	.018	.064	-.105	1	.284**	.166**	-.010	.233*	.176**	.214**	.048	.120*	.046
	Sig. (2-tailed)	.167	.748	.276	.064		.000	.003	.880	.016	.002	.006	.382	.027	.435
	N	339	315	291	314	339	335	329	222	107	305	163	339	339	289

IL-10 plasma levels (pg/ml)	Pearson Correlation	.124*	.033	-.035	-.154**	.284**	1	.143**	.006	.169	.208**	.134	.173**	.122*	.107
	Sig. (2-tailed)	.022	.563	.557	.006	.000		.010	.926	.080	.000	.089	.001	.024	.069
	N	339	315	291	313	335	339	328	221	108	305	163	339	339	290
IL-12p70 plasma levels (pg/ml)	Pearson Correlation	.151**	-.090	.077	-.089	.166**	.143**	1	.281**	.134	.221**	.054	.013	.199**	.064
	Sig. (2-tailed)	.006	.112	.198	.120	.003	.010		.000	.176	.000	.497	.817	.000	.283
	N	332	311	285	307	329	328	332	220	103	298	162	332	332	286
IL-13 plasma levels (pg/ml)	Pearson Correlation	.105	.034	.121	-.140*	-.010	.006	.281**	1	.292*	.160*	.073	.062	.322**	.141
	Sig. (2-tailed)	.117	.629	.093	.043	.880	.926	.000		.010	.023	.464	.358	.000	.053
	N	225	208	193	209	222	221	220	225	76	202	102	225	225	190
IL-1β plasma levels (pg/ml)	Pearson Correlation	.061	.122	.157	-.195*	.233*	.169	.134	.292*	1	.222*	.177	.220*	.275**	.333**
	Sig. (2-tailed)	.525	.223	.120	.050	.016	.080	.176	.010		.025	.204	.021	.004	.002
	N	110	102	99	102	107	108	103	76	110	102	53	110	110	86
IL-2 plasma levles (pg/ml)	Pearson Correlation	.196**	-.045	.061	-.127*	.176**	.208**	.221**	.160*	.222*	1	.126	.073	.162**	.012
	Sig. (2-tailed)	.001	.447	.323	.033	.002	.000	.000	.023	.025		.130	.204	.004	.850
	N	308	285	266	283	305	305	298	202	102	308	145	308	308	264
IL-4 plasma levels (pg/ml)	Pearson Correlation	-.074	.039	.007	-.020	.214**	.134	.054	.073	.177	.126	1	.018	.011	-.027
	Sig. (2-tailed)	.347	.630	.934	.807	.006	.089	.497	.464	.204	.130		.821	.888	.741
	N	163	159	141	151	163	163	162	102	53	145	163	163	163	152
	Pearson Correlation	.456**	.225**	.020	-.200**	.048	.173**	.013	.062	.220*	.073	.018	1	.264**	.372**

IL-6 plasma levels (pg/ml)	Sig. (2-tailed)	.000	.000	.737	.000	.382	.001	.817	.358	.021	.204	.821		.000	.000
	N	343	319	295	317	339	339	332	225	110	308	163	343	343	293
IL-8 plasma levels (pg/ml)	Pearson Correlation	.368**	.089	.122*	-.234**	.120*	.122*	.199**	.322**	.275**	.162**	.011	.264**	1	.298**
	Sig. (2-tailed)	.000	.114	.037	.000	.027	.024	.000	.000	.004	.004	.888	.000		.000
	N	343	319	295	317	339	339	332	225	110	308	163	343	343	293
CRP levels (mg/dl)	Pearson Correlation	.101	.073	-.111	-.115	.046	.107	.064	.141	.333**	.012	-.027	.372**	.298**	1
	Sig. (2-tailed)	.084	.229	.079	.059	.435	.069	.283	.053	.002	.850	.741	.000	.000	
	N	294	272	251	271	289	290	286	190	86	264	152	293	293	294

BMI: body mass index, BP: blood pressure, IFN-γ: interferon gamma, IL-10: interleukin 10, IL-12p70: interleukin 12p70; IL-13: interleukin 13; IL-1β: interleukin 1 beta; IL-2: interleukin 2; IL-4: interleukin 4; IL-6: interleukin6; IL-8: interleukin 8; CRP: C reactive protein.

*** Correlation is significant at the 0.01 level (2-tailed).*

** Correlation is significant at the 0.05 level (2-tailed)*

<u>Table 7:</u> Biomarker levels associated with MACE within one year of admission.

Protein	**Protein name**	**No MACE (n=213)**	**MACE (n=16)**	*p value*	**MACE within 6 months (n=5)**	**MACE after 6 months (n=11)**	*p value*
		mean ± SD	*mean ± SD*		*mean ± SD*	*mean ± SD*	
TACE mRNA levels/GAPDH (2^(-DcT)	Tumour necrosis factor alpha converting enzyme	0.0540 ± 0.03150	0.0485 ± 0.04828	*0.538*	0.0495 ± 0.0258	0.4814 ± 0.0308	*0.798*
Plasma levels (pg/ml)							
TACE	Tumour necrosis factor alpha converting enzyme	684.73 ± 2413.31	1472.15 ± 5574.62	*0.525*	99.68 ± 161.66	2096.00 ± 6725.63	*0.801*
TNFα	Tumour necrosis factor alpha	4.661 ± 2.483	6.364 ± 1.943	*0.559*	6.118 ± 0.716	5.022 ± 2.246	*0.292*
TNFR1	Tumour necrosis factor alpha receptor 1	1237.65 ± 1121.52	2003.62 ± 142.50	*0.018*	1708.04 ± 930.91	2137.97 ± 1618.90	*0.061*
TNFR2	Tumour necrosis factor alpha receptor 2	2929.10 ± 2219.55	3525.37 ± 1879.69	*0.348*	3847.00 ± 1939.68	3379.18 ± 1928.64	*0.453*
TIMP3	Metalloproteinase inhibitor 3	3420.52 ± 2702.14	3085.77 ± 2214.90	*0.589*	3842.40 ± 3333.99	2741.85 ± 1579.92	*0.624*
IFN-γ	Interferon gamma	9.61 ± 41.23	7.22 ± 7.50	*0.942*	*5.00 ± 2.37*	8.22 ± 8.86	*0.852*
IL-10	Interleukin 10	0.37 ± 0.60	0.42 ± 0.63	*0.631*	0.28 ± 0.10	0.48 ± 0.75	*0.886*
IL-12p70	Interleukin 12 p70	0.31 ± 0.35	0.22 ± 0.14	*0.148*	0.21 ± 0.13	0.22 ± 0.16	*0.351*
IL-13	Interleukin 13	0.79 ± 0.63	0.64 ± 0.25	*0.775*	0.65 ± 0.38	0.63 ± 0.21	*0.949*
IL-1β	Interleukin 1 beta	0.41 ± 1.09	0.35 ± 0.13	*0.323*	0.26 ± 0.09	0.41 ± 0.13	*0.543*
IL-2	Interleukin 2	0.27 ± 0.28	0.26 ± 0.15	*0.938*	0.24 ± 0.16	0.28 ± 0.16	*0.836*
IL-4	Interleukin 4	0.03 ± 0.15	0.02 ± 0.01	*0.74*	0.02 ± 0.01	0.01 ± 0.01	*0.763*
IL-6	Interleukin 6	1.99 ± 2.80	3.06 ± 2.67	*0.021*	4.66 ± 3.78	2.33 ± 1.77	*0.023*
IL-8	Interleukin 8	8.22 ± 10.35	7.40 ± 2.63	*0.452*	7.68 ± 2.82	7.28 ± 2.66	*0.741*

Protein	**Protein name**	**no MACE (n=192)**		**MACE (n=16)**		*p value* ***(ranked by p value)***	**MACE within 6 months (n=5)**		**MACE after 6 months (n=11)**		*p value*
		Mean (NPX)	*Std. deviation*	*Mean (NPX)*	*Std. deviation*		*Mean (NPX)*	*Std. deviation*	*Mean (NPX)*	*Std. deviation*	
RARRES2	Retinoic acid receptor responder protein 2	10.517448	0.3209137	**10.816250**	0.2389386	0.000	10.730000	0.1862794	**10.855455**	0.2575796	*.001*
CCL16	C-C motif chemokine 16	5.916198	0.5037531	**6.378125**	0.4575474	0.000	6.074000	0.3990363	**6.516364**	0.4278615	*.001*
IL_1RT1	Interleukin-1 receptor type 1	5.522917	0.3008937	**5.845625**	0.2807364	0.001	5.822000	0.1693222	**5.856364**	0.3261065	*.005*
TNF_R1	Tumour necrosis factor alpha receptor 1	5.741771	0.5985637	**6.395625**	0.7150661	0.001	6.242000	0.6036307	**6.465455**	0.7772176	*.005*
TFF3	Trefoil factor 3	5.076510	0.6515530	**5.811875**	0.7697291	0.002	5.512000	0.5859778	**5.948182**	0.8282369	*.004*
ADM	Adrenomedullin	7.023385	0.5045712	**7.592500**	0.5457533	0.003	7.386000	0.5586412	**7.686364**	0.5393751	*.006*
CXCL16	C-X-C chemokine motif 16	4.520104	0.3406999	**4.785000**	0.3669332	0.003	4.600000	0.1437011	**4.869091**	0.4108638	.005
TR	Transferrin receptor protein 1	3.716042	0.5969126	**4.179375**	0.7781642	0.004	**4.284000**	0.5000300	4.131818	0.8946152	.014
ADAM_TS13	A disintegrin and metalloproteinase with thrombospondin motifs 13	**5.338281**	0.2131186	5.179375	0.1636039	0.004	5.178000	0.1821263	5.180000	0.1639512	.016
TRAIL_R2	TNF-related apoptosis-inducing ligand receptor 2	5.491250	0.5557971	**6.057500**	0.7003285	0.005	5.900000	0.6702238	**6.129091**	0.7334638	*.016*
MMP_3	Matrix metalloproteinase 3	5.542344	0.7262550	**6.229375**	0.9879775	0.007	5.948000	0.7238232	**6.357273**	1.0940758	*.017*
LTBR	Lymphotoxin-beta receptor	2.585938	0.4730907	**3.010000**	0.4421010	0.008	2.998000	0.4855615	**3.015455**	0.4458332	*.028*
TNFRSF14	Tumour necrosis factor receptor superfamily member 14	3.651823	0.5276961	**4.136875**	0.4769447	0.008	**4.156000**	0.2433721	4.128182	0.5632551	*.028*

PD_L2	Programmed cell death 1 ligand 2	2.550052	0.3559435	**2.852500**	0.2421707	0.008	2.684000	0.2176695	**2.929091**	0.2199297	*.014*
GDF_15	Growth/differentiation factor 15	4.718125	0.7409981	**5.460000**	0.6679820	0.010	**5.490000**	0.7614132	5.446364	0.6608672	.035
EPHB4	Ephrin type-B receptor 4	1.133802	0.3035697	**1.425000**	0.3382110	0.010	1.368000	0.3525195	**1.450909**	0.3457009	.035
IL2_RA	Interleukin-2 receptor subunit alpha	3.589479	0.4941954	**4.014375**	0.4609695	0.011	3.928000	0.4851495	**4.053636**	0.4681511	.036
SHPS_1	Tyrosine-protein phosphatase non-receptor type substrate 1	2.754375	0.4158793	**3.031875**	0.3988019	0.011	3.016000	0.4170492	**3.039091**	0.4108638	.039
IDUA	Alpha-L-iduronidase	**6.080729**	0.4762775	5.763750	0.4533560	0.011	5.892000	0.2622403	5.705455	0.5184856	.030
AGRP	Agouti related protein	4.057344	0.4433638	**4.341250**	0.4572800	0.013	**4.386000**	0.4132554	4.320909	0.4938513	.043
IL_1RT2	Interleukin 1 receptor 2	4.319896	0.3453036	**4.499375**	0.4784066	0.013	4.350000	0.2610555	**4.567273**	0.5475599	.025
CCL22	C-C motif chemokine 22	**2.633125**	1.1412919	2.183750	1.0883497	0.013	2.292000	0.9146420	2.134545	1.1973334	.034
KLK6	Kallikrein-6	5.955260	0.3643569	**6.236250**	0.3104593	0.013	6.184000	0.2272224	**6.260000**	0.3491991	.045
THBS2	Thrombospondin 2	6.061094	0.2761769	**6.286250**	0.3642687	0.014	**6.398000**	0.2672452	6.235455	0.4017303	*.026*
IL_4RA	Interleukin-4 receptor subunit alpha	1.848542	0.3232329	**2.118125**	0.4397760	0.014	2.060000	0.3672874	**2.144545**	0.4834121	.047
PI3	Elafin	4.721250	0.7060045	**5.249375**	0.5761304	0.016	**5.396000**	0.5717342	5.182727	0.5928759	.046
PLC	Perlecan	5.714219	0.4971557	**6.153750**	0.4536941	0.016	6.078000	0.3624500	**6.188182**	0.5020322	.053
REN	Renin	7.823073	0.9995300	**8.598750**	1.0321555	0.021	8.030000	1.0926802	**8.857273**	0.9408410	*.023*
IL_6	Interleukin 6	3.586094	1.0389442	**4.356875**	0.9402887	0.021	**5.040000**	0.9692523	4.046364	0.7816940	*.011*
VSIG2	V-set and immunoglobulin domain-containing protein 2	2.767344	0.7471024	**3.365625**	0.6385814	0.022	**3.436000**	0.9647435	3.333636	0.4855774	.051
IGFBP_2	Insulin-like growth factor-binding protein 2	6.937344	0.8024718	**7.675000**	0.6414255	0.023	7.630000	0.4454773	**7.695455**	0.7323164	0.075

U_PAR	Urokinase plasminogen activator surface receptor	4.442448	0.4484432	**4.800625**	0.4336584	0.023	4.656000	0.3357529	**4.866364**	0.4709410	.055
FGF_23	Fibroblast growth factor 23	2.911563	0.9305224	**3.471875**	1.2912383	0.026	2.996000	0.7663746	**3.688182**	1.4495918	.035
AMBP	Protein Alpha-1-Microglobulin/Bikunin Precursor	6.017760	0.2140722	**6.185625**	0.1927336	0.030	5.978000	0.1728294	**6.280000**	0.1114451	*.003*
CTSD	Cathepsin D	4.717344	0.4094073	**4.783750**	0.4192831	0.033	**4.900000**	0.5829666	4.730909	0.3434081	0.617
RAGE	Receptor for advanced glycosylation end products	5.253073	0.4411164	**5.591250**	0.5084339	0.033	5.564000	0.2802320	**5.603636**	0.5964943	0.104
TNF_R2	Tumour necrosis factor alpha receptor 2	4.019948	0.5368265	**4.472500**	0.5676090	0.034	4.328000	0.5289329	**4.538182**	0.5968051	0.089
GRN	Granulins	5.808229	0.3162112	**5.983750**	0.3159087	0.034	5.884000	0.2644428	**6.029091**	0.3383624	0.074
CD93	Complement component C1q receptor	8.498125	0.3812608	**8.778750**	0.3784860	0.036	8.614000	0.2192715	**8.853636**	0.4194108	*.060*
PAR_1	Proteinase activated receptor 1	8.195365	0.4114206	**8.476250**	0.3283266	0.036	**8.614000**	0.2005742	8.413636	0.3630502	.069
SPON1	Spondin 1	0.617760	0.2683451	**0.853125**	0.3129796	0.038	0.816000	0.2666083	**0.870000**	0.3427827	0.115
ST2	Interleukin 1 receptor-like 1	3.273125	0.7503149	**3.790000**	0.8300040	0.041	**3.812000**	0.4671937	3.780000	0.9724711	0.123
FGF_21	Fibroblast growth factor 21	6.477656	1.6182280	**7.473750**	1.2729801	0.042	6.888000	1.6344479	**7.740000**	1.0548080	0.080
CPA1	Carboxypeptidase 1	4.556667	0.7487577	**4.952500**	0.8252555	0.045	**4.990000**	0.6685806	4.935455	0.9174679	0.134
CCL15	C-C motif chemokine 15	5.960052	0.5879759	**6.367500**	0.6057557	0.045	6.140000	0.5639592	**6.470909**	0.6209260	0.083
IGFBP_7	Insulin like growth factor binding protein 7	3.425990	0.4556648	**3.764375**	0.3919518	0.048	**3.790000**	0.1959592	3.752727	0.4632514	0.137
IL1RL2	Interleukin 1 receptor like 2	**3.163802**	0.3820825	2.960625	0.5209155	0.049	3.152000	0.3006160	2.873636	0.5867243	.061

Highlighted values refer to the highest value among the compared groups. MACE: major adverse cardiovascular events; VHR: very high risk.

<u>Table 8:</u> Sensitivity and specificity of the measured biomarkers in differentiating between VHR and non-VHR individuals

	Protein levels			ROC							
	VHR	**Non-VHR**	***p value (VHR vs. non-VHR)***	**Cut-off value (pg/ml)**	**Sensitivity**	**Specificity**	**AUC**	**Std error**	***p value***	**CI**	
Number of patients	229	115									
Plasma levels (pg/ml)										**Lower CI**	**Upper CI**
CRP (mg/L)	*12.41 ± 32.48*	*2.54 ± 3.45*	***0.008***	**3.0**			**0.667**	**0.032**	*<0.0001*	**0.605**	**0.729**
TNFα	4.711 ± 2.453	2.770 ± 0.752 (n=113)	<0.0001	3.08	0.80	0.71	0.835	0.022	<0.0001	0.793	0.877
TACE	739.74 ± 2738.67	905.90 ± 3750.23	0.642	N/A							
TNFR1	1291 ± 1158	817.4 ± 208.6	0.006	847.45	0.77	0.69	0.785	0.026	<0.0001	0.734	0.835
TNFR2	2971.0 ± 2199.0	2133.0 ± 614.0	0.057	2102.50	0.71	0.58	0.711	0.028	<0.0001	0.656	0.767
TIMP3	3397.0 ± 2668.0	3144.0 ± 2516.0	0.486	N/A							
IFN-γ	9.93 ± 42.02	8.22 ± 11.43	0.003	N/A			0.446	0.032	0.105	0.384	0.508
IL-10	0.375 ± 0.600	0.358 ± 0.990	0.39	N/A							
IL-12p70	0.299 ± 0.345	0.220 ± 0.155	0.084	N/A							
IL-13	0.782 ± 0.611	0.625 ± 0.542	0.05	N/A							
IL-1β	0.407 ± 1.052	0.185 ± 0.120	0.111	N/A							
IL-2	0.266 ± 0.275	0.231 ± 0.272	0.775	N/A							
IL-4	0.0323 ± 0.1451	0.018 ± 0.013	0.395	N/A							
IL-6	2.06 ± 2.80	0.066 ± 0.512	<0.0001	0.66	0.80	0.68	0.813	0.025	<0.0001	0.764	0.861
IL-8	8.16 ± 10.00	3.50 ± 1.99	<0.0001	4.06	0.75	0.69	0.804	0.024	<0.0001	0.756	0.851
Number of patients	208	89	***p value***	**Cut-off value NPX**	**Sensitivity**	**Specificity**	**AUC**	**Sd error**	***p value***	**Lower CI**	**Upper CI**
Levels expressed in NPX values (means)											
GDF_15	4.78	3.87	0.002	4.07	0.85	0.81	0.883	0.022	<0.0001	0.841	0.926
TRAIL_R2	5.53	4.9	<0.0001	5.09	0.80	0.78	0.868	0.022	<0.0001	0.824	0.911
MMP_7	9.57	8.72	<0.0001	8.98	0.85	0.71	0.865	0.024	<0.0001	0.818	0.911
MMP_12	7.82	6.95	<0.0001	7.14	0.80	0.66	0.825	0.024	<0.0001	0.778	0.873
U_PAR	4.47	4.05	<0.0001	4.15	0.76	0.71	0.814	0.026	<0.0001	0.764	0.865
REN	7.88	6.87	0.001	7.12	0.81	0.72	0.809	0.026	<0.0001	0.758	0.859
IL_18BP	4.89	4.47	0.003	4.60	0.75	0.72	0.797	0.027	<0.0001	0.744	0.850

CEACAM8	3.72	3.16	<0.0001	3.36	0.73	0.72	0.796	0.027	<0.0001	0.744	0.848
ACE2	4.1	3.5	<0.0001	3.59	0.76	0.70	0.795	0.030	<0.0001	0.735	0.855
PTX3	3.27	2.77	<0.0001	3.01	0.74	0.71	0.785	0.027	<0.0001	0.732	0.838
TNF_R1	5.79	5.34	0.05	5.42	0.75	0.71	0.778	0.029	<0.0001	0.721	0.836
EGFR	***2.16***	***2.36***	0.012	2.29	0.65	0.71	0.773	0.030	<0.0001	0.715	0.831
TR_AP	4.47	4.08	<0.0001	4.29	0.71	0.69	0.770	0.030	<0.0001	0.711	0.829
SPON1	0.64	0.43	0.012	0.48	0.69	0.72	0.764	0.028	<0.0001	0.709	0.818
t_PA	5.66	5.14	0.067	5.37	0.70	0.69	0.761	0.029	<0.0001	0.705	0.816
CSTB	5.07	4.6	0.034	4.74	0.68	0.71	0.755	0.030	<0.0001	0.697	0.813
TIM	7.39	6.68	0.067	6.82	0.71	0.66	0.752	0.032	<0.0001	0.689	0.815
CTSD	4.72	4.44	0.005	4.49	0.71	0.70	0.751	0.033	<0.0001	0.686	0.816
PSP_D	1.69	1.1	0.003	1.26	0.71	0.66	0.750	0.031	<0.0001	0.690	0.809
CCL3	3.66	3.3	0.062	3.41	0.66	0.66	0.746	0.032	<0.0001	0.683	0.808
OPN	4.12	3.59	0.015	3.70	0.70	0.64	0.745	0.029	<0.0001	0.689	0.801
hOSCAR	10.44	10.21	<0.0001	10.35	0.69	0.72	0.745	0.031	<0.0001	0.684	0.805
IL2_RA	3.62	3.24	0.005	3.41	0.63	0.72	0.741	0.029	<0.0001	0.683	0.798
TNFRSF10A	2.39	2.1	<0.0001	2.19	0.69	0.71	0.738	0.032	<0.0001	0.676	0.799
CD4	2.92	2.63	0.001	2.71	0.67	0.66	0.737	0.031	<0.0001	0.676	0.798
MMP_9	3.78	3.3	<0.0001	3.59	0.63	0.71	0.736	0.029	<0.0001	0.680	0.792
CHIT1	5.45	4.63	0.054	5.08	0.70	0.70	0.735	0.031	<0.0001	0.674	0.795
TNFRSF11A	5.01	4.59	0.023	4.68	0.65	0.65	0.734	0.031	<0.0001	0.674	0.794
PON3	***5.04***	***5.53***	0.011	5.34	0.66	0.70	0.725	0.034	<0.0001	0.658	0.792
LOX_1	6.2	5.83	<0.0001	5.94	0.66	0.65	0.724	0.030	<0.0001	0.665	0.784
MCP_1	2.63	2.37	0.016	2.41	0.71	0.64	0.723	0.031	<0.0001	0.663	0.784
CNTN1	***1.72***	***1.93***	<0.0001	1.83	0.67	0.64	0.723	0.031	<0.0001	0.663	0.783
Gal_9	7.8	7.54	0.041	7.59	0.66	0.65	0.723	0.032	<0.0001	0.659	0.787
CTSZ	4.74	4.43	0.041	4.60	0.62	0.71	0.722	0.032	<0.0001	0.660	0.785
PlGF	7.76	7.35	0.003	7.20	0.70	0.65	0.722	0.032	<0.0001	0.660	0.785
IL_4RA	1.87	1.66	0.002	1.74	0.62	0.72	0.718	0.032	<0.0001	0.655	0.781
AZU1	3.07	2.65	<0.0001	2.76	0.68	0.65	0.709	0.032	<0.0001	0.646	0.772
Gal_4	2.91	2.52	0.032	2.62	0.65	0.64	0.706	0.032	<0.0001	0.644	0.769
CD163	6.16	5.88	0.015	5.95	0.65	0.64	0.685	0.034	<0.0001	0.617	0.752
Gal_3	5.00	4.83	0.007	4.85	0.66	0.58	0.671	0.033	<0.0001	0.606	0.737
GLO1	6.55	6.23	<0.0001	6.33	0.63	0.62	0.664	0.034	<0.0001	0.597	0.730
vWF	5.34	4.87	0.005	4.98	0.61	0.63	0.660	0.032	<0.0001	0.597	0.723
FABP4	5.07	4.6	0.006	4.69	0.63	0.61	0.656	0.034	<0.0001	0.589	0.723
RARRES2	10.54	10.37	0.012	10.44	0.65	0.56	0.652	0.035	<0.0001	0.584	0.720
LPL	***9.41***	***9.64***	0.007	9.59	0.66	0.63	0.647	0.033	<0.0001	0.582	0.713
ICAM_2	4.03	3.85	0.051	3.88	0.68	0.51	0.640	0.034	<0.0001	0.573	0.706
MMP_3	5.6	5.26	0.033	5.29	0.62	0.56	0.639	0.037	<0.0001	0.567	0.712
IDUA	6.06	5.84	0.008	5.86	0.68	0.51	0.638	0.034	<0.0001	0.572	0.705
RETN	5.93	5.65	0.052	5.68	0.65	0.51	0.637	0.034	<0.0001	0.571	0.703
FGF_21	6.55	5.79	0.004	6.10	0.61	0.57	0.631	0.036	<0.0001	0.561	0.701
HAOX1	4.31	3.79	0.008	3.69	0.61	0.60	0.631	0.035	<0.0001	0.562	0.700
MPO	3.22	3.08	0.057	3.12	0.65	0.63	0.623	0.035	0.001	0.554	0.691
PRTN3	5.04	4.83	0.001	4.81	0.66	0.51	0.621	0.034	0.001	0.554	0.688
ITGB2	***4.14***	***4.29***	0.043	4.18	0.63	0.60	0.619	0.035	0.001	0.550	0.689
PD_L2	2.57	2.45	0.043	2.48	0.63	0.56	0.616	0.035	0.002	0.547	0.685
PRELP	6.33	6.27	0.001	6.24	0.66	0.51	0.610	0.034	0.003	0.543	0.678
IL_1ra	6.92	6.66	0.009	6.65	0.56	0.52	0.609	0.037	0.003	0.535	0.682
PCSK9	0.92	0.82	0.003	0.81	0.65	0.53	0.605	0.036	0.004	0.534	0.676
IL1RL2	3.15	3	0.011	3.04	0.62	0.51	0.603	0.036	0.005	0.533	0.672
SRC	***7.02***	***7.3***	0.036	7.32	0.55	0.55	0.602	0.035	0.005	0.532	0.671
DECR1	5.9	5.55	0.037	5.77	0.54	0.65	0.601	0.034	0.006	0.534	0.667
JAM_A	4.86	4.63	0.022	4.56	0.62	0.52	0.594	0.035	0.010	0.525	0.664
LEP	***5.18***	***5.25***	0.055	5.26	0.62	0.51	0.572	0.038	0.050	0.497	0.647
MMP_2	***3.57***	***3.62***	<0.0001	3.54	0.65	0.52	0.562	0.035	0.093	0.492	0.631
HSP_27	***10.2***	***10.26***	0.028	10.22	0.64	0.50	0.561	0.034	0.095	0.495	0.627

LDL receptor	2.85	2.77	0.008	2.71	0.60	0.51	0.560	0.039	0.103	0.484	0.635
Notch_3	2.45	2.37	0.012	2.39	0.57	0.51	0.559	0.035	0.107	0.491	0.627
IGFBP_1	***2.97***	***3.15***	0.011	3.05	0.56	0.53	0.558	0.035	0.115	0.489	0.627
CD84	5.00	4.91	0.028	4.91	0.55	0.51	0.547	0.035	0.199	0.479	0.615
SCF	***8.61***	***8.69***	0.009	8.74	0.52	0.51	0.528	0.036	0.449	0.458	0.598
AXL	***6.53***	***6.55***	0.03	6.48	0.51	0.48	0.496	0.038	0.911	0.421	0.571

Receiver operating curves (ROC) were calculated for proteins that were able to differentiate between VHR and non-VHR patients. Proteins were then ranked according to the AUC value. Cut off values refer to the protein level threshold for differentiating between VHR and the non-VHR cohort. Sensitivity refers to the true positive participants which is the probability of a VHR participant to have high levels of the protein in question. Specificity refers to the false positive participants which is the proportion of non-VHR participants who have a low level of the protein in question. Sensitivity and specificity are expressed in ratios. The Area Under the Curve (AUC) is the test's ability to reliably distinguish between VHR and non-VHR patients. An AUC equal to 1 represents a test with an excellent sensitivity and specificity. Values in bold and italic refer to the proteins which levels were higher in non-VHR patients. The confidence interval represents the AUC interval of 95% of the observations. CI: confidence interval; non-VHR: non-very high risk; NPX: normalised protein ratio, ROC: receiver operating characteristics VHR: very high risk

IV. Literature Review

Role of tumour necrosis factor alphaconverting enzyme (TACE/ADAM17) andassociated proteins in coronary arterydisease and cardiac events

Chemaly M, McGilligan V, Gibson M, Clauss M, Watterson S, Alexander HD, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis. 2017 Dec;110(12):700–11.

Archives of Cardiovascular Disease (2017) xxx, xxx—xxx

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com/en

REVIEW

Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events

Rôle de l'enzyme de conversion TNF (TACE/ADAM17) et des protéines associées dans la maladie coronaire et les événements cardiaques

Melody Chemaly [a,*], Victoria McGilligan [a], Mark Gibson [b], Matthias Clauss [c,d], Steven Watterson [a], H. Denis Alexander [a], Anthony John Bjourson [a], Aaron Peace [a,b]

[a] *Northern Ireland centre for stratified medicine, school of biomedical sciences, Ulster university, CTRIC building, Altnagelvin hospital, Glenshane Road, BT47 6SB Londonderry, Northern Ireland*
[b] *Cardiology department, Western health and social care trust, Altnagelvin hospital, Glenshane road, BT47 6SB Londonderry, Northern Ireland*
[c] *Department of cellular and integrative physiology, Indiana university, Indianapolis, 46202 Indiana, USA*
[d] *Centre for molecular bioscience, Ulster university, BT52 1SA Coleraine, Northern Ireland*

Received 5 June 2017; received in revised form 13 August 2017; accepted 16 August 2017

KEYWORDS
Atherosclerosis;
Transmembrane proteins;
TNFα

Summary Tumour necrosis factor alpha converting enzyme (TACE/ADAM17) is a member of the A disintegrin and metalloproteinase (ADAM) family of ectodomain shedding proteinases. It regulates many inflammatory processes by cleaving several transmembrane proteins, including tumour necrosis factor alpha (TNFα) and its receptors tumour necrosis factor alpha receptor 1 and tumour necrosis factor alpha receptor 2. There is evidence that TACE is involved in several

Abbreviations: ACS, Acute coronary syndrome; AMI, Acute myocardial infarction; CAD, Coronary artery disease; MACE, Major adverse cardiac events; MAPK, Mitogen-activated protein kinases; TACE (CD156b), Tumour necrosis factor alpha converting enzyme or A disintegrin and metalloproteinase 17 (ADAM17); TIMP3, Tissue inhibitor of metalloproteinase 3; TNFα, Tumour necrosis factor alpha; TNFR, TNF receptor.

* Corresponding author. Northern Ireland centre for stratified medicine, school of biomedical sciences, Ulster university, CTRIC building, Altnagelvin hospital, BT47 6SB Londonderry, Northern Ireland.
E-mail address: el_chemaly-m@ulster.ac.uk (M. Chemaly).

https://doi.org/10.1016/j.acvd.2017.08.002

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

Biomarker;
TACE

inflammatory diseases, such as ischaemia, heart failure, arthritis, atherosclerosis, diabetes and cancer as well as neurological and immune diseases. This review summarizes the latest discoveries regarding the mechanism of action and regulation of TACE. It also focuses on the role of TACE in atherosclerosis and coronary artery disease (CAD), highlighting clinical studies that have investigated its expression and protein activity. The multitude of substrates cleaved by TACE make this enzyme an attractive target for therapy and a candidate for biomarker research and development in CAD.

MOTS CLÉS
Athérosclérose ;
Protéines transmembrannaires ;
TNFα ;
Biomarqueur ;
TACE

Résumé L'enzyme de conversion du facteur alpha de nécrose tumorale (TACE/ADAM17), membre de la famille des A désintégrines et métalloprotéinases (ADAM) qui sont des protéinases clivant l'ectodomaine des protéines transmembrannaires, régule différents processus inflammatoires en clivant des protéines transmembrannaires, y comrpis le facteur alpha de nécrose tumorale (TNFα) et ses récepteurs 1 et 2. Différentes études ont montré l'association de TACE avec des maladies inflammatoires tel que l'ischémie, l'insuffisance cardiaque, l'arthrite, l'athérosclérose, le diabète, le cancer ainsi que des maladies neurologiques et immunologiques. Cette revue résume les dernières découvertes concernant le mécanisme d'action et de régulation de TACE ainsi que son rôle dans l'athérosclerose et les maladies coronariennes en mettant en évidence les études cliniques les plus récentes en relation avec son expression et son activité. La multitude des substrats clivés par TACE rendent cette enzyme une cible thérapeutique intéressante surtout dans le domaine du développement des biomarqueurs pour les maladies coronariennes.

Background

Tumour necrosis factor alpha converting enzyme (TACE), also known as A disintegrin and metalloproteinase 17 (ADAM17), is a membrane-anchored protein responsible for the ectodomain shedding of a variety of transmembrane proteins, such as cytokines, chemokines, and growth factors and their receptors. Shedding results in the initiation or inhibition of downstream signalling and cellular responses, and is associated with several major acute and chronic inflammatory diseases. Recent studies have reported overexpression of TACE in patients with coronary artery disease (CAD) as well as after acute myocardial infarction (AMI), indicating that TACE may be a useful cardiac prognostic biomarker of cardiac events. This review summarizes recent findings on TACE activity and regulation, with an emphasis on the role of TACE in CAD.

Structure

Black et al. first described TACE in 1997 when working with mammalian THP1 cells, as the enzyme that cleaves tumour necrosis factor alpha (TNFα), and reported purification and cloning of the protein [1]. Subsequently, different forms of TACE have been described including the full-length protein (~110 KDa under non-reducing conditions), a mature form of TACE lacking the prodomain (80 KDa), and a third form detected in cell lysates, which lacks the cytoplasmic domain (60 KDa) [2] (Fig. 1).

Localization

Immunohistochemical studies suggest that most of the active form of TACE is localized in the cellular perinuclear region, with a small amount present on the plasma membrane surface [2]. Tellier et al. further reported that TACE is sequestered into lipid rafts (Fig. 2). This spatial distribution has a role in the regulation of TACE activity by keeping the enzyme separate from its substrates [3].

Lipid rafts are known to have high concentrations of cholesterol, and interestingly, the shedding of TACE substrates, such as CD30 [4], interleukin-6 receptor (IL-6R) [5] and L-selectin (CD62L) [6], can be increased by cholesterol-lowering drugs. The increase in TACE shedding was also observed with TNFα, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) after membrane cholesterol depletion [3]. Disruption of the lipid rafts may displace the mature form of TACE in the non-raft region of the membrane that contains the major part of TACE substrates, and increase their shedding. Therefore, under normal conditions, the sequestration of the mature form of TACE in lipid rafts can be considered as the rate-limiting process of its shedding activity [7].

Activation and regulation

Tissue inhibitor of metalloproteinase 3 (TIMP3) is the only known endogenous inhibitor of TACE [8].

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

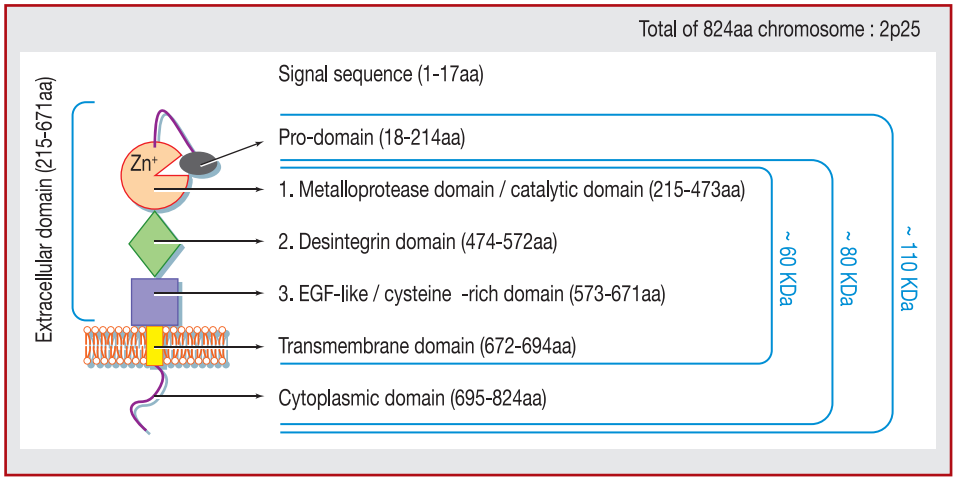


Figure 1. TACE domains. Signal peptide (1-17 aa); pro-domain (18-214 aa) acting as an inactivator and a chaperone domain; extracellular domain (215-671 aa) comprising: 1. A metalloprotease domain/catalytic domain (215-473 aa) responsible for an ectodomain shedding; 2. A disintegrin domain (474-572 aa); 3. An EGF-like/cysteine rich domain (573-671 aa) responsible for substrate recognition and activation; transmembrane domain (672-694 aa) necessary for effective cleavage of substrates; cytoplasmic domain (695-824 aa) binding to many proteins that regulate TACE activity. aa: amino acid; EGF: endothelial growth factor; TACE: tumour necrosis factor alpha converting enzyme.

TIMP3 is downregulated in circulating human monocytes in people at high risk of diabetes and atherosclerosis [9]. Stöhr et al. demonstrated that TIMP3 also regulates lipid metabolism as well as the oxidative stress response, maintaining metabolic flexibility in the heart, particularly during episodes of increased cardiac stress [10]. TIMP3 over expression has been shown to improve post-myocardial infarction cardiac remodelling related to lower extracellular matrix disruption in animal models [11,12].

Regarding TIMP3 activation, Cesaro et al. showed that strong TACE expression was associated with early acute phase inflammation in Crohn's disease, whereas TIMP3 was upregulated during the quiescent phase of the disease [13]. TIMP3 may therefore be involved in a delayed regulatory mechanism following an increased TACE expression, and its role in inflammation and heart related diseases should be studied further.

Studies have shown that only the monomeric form of TACE is active and can effectively cleave its substrates. However, TACE appears to be predominantly present as dimers at the cell surface, which enables its efficient association with TIMP3 and silences its activity. Hence, TIMP3 inhibits TACE only when it is in its dimer form. Upon activation of the p38 mitogen-activated protein kinase pathway, the balance can shift from TACE dimers to monomers, and this shift is associated with an increase in cell surface presentation of TACE and a reduction in TIMP3 association [14].

Substrates and shedding process

TACE mediates cell-cell interactions with a wide range of identified substrates, although the mechanisms and consequences of this binding are not yet fully understood. As the sequences cleaved in various substrates are highly variable, there is no apparent consensus for the TACE cleavage sequence. New evidence suggests that TACE activity is regulated by its non-catalytic domains and the secondary structure of its substrates [15].

Cleavage of TACE substrates occurs at extracellular sites proximal to the cell membrane, thereby releasing the soluble ectodomain from the cell surface. The cleaved molecules can then bind to their receptor on the same cell (autocrine effect) or to receptors on neighbouring cells (paracrine effect) or even enter the bloodstream (endocrine effect). When the substrate is cleaved and bound to its receptor, it can initiate downstream signalling events. Alternatively, the receptor can also be cleaved from the cell surface; thus, ectodomain shedding can actually stop the ligand-initiated signalling.

Because of its many functional properties, TACE plays a major role in inflammation through its shedding of a variety of inflammatory substrates. Studies have shown that TACE is implicated in platelet function through its cleavage of the von Willebrand factor (CD42b) receptor [16]. TACE is also responsible for the cleavage of L-selectin (CD62L), intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) [17]. Many TACE substrates have been extensively investigated in atherosclerosis, and all are known to participate in the inflammatory process accompanying the formation and progression of plaque (Fig. 3). The consequences of TACE shedding on these factors needs to be closely investigated, as their soluble forms may hold different properties compared to their membrane forms. For example, the cleavage of TNFR1 by TACE sheds a soluble form of TNFR1 that binds to free TNFα, dampening the inflammatory response [18]. In addition, TACE activates ligands (such as neuregulin) that bind to the ERbB tyrosine kinase family of receptors. The resulting signalling pathways have been involved in the growth of many tumour types as well as the maintenance of cardiac function [19,20].

The fate of TACE after its activation

There appears to be contradiction in the understanding what happens to TACE after it has been activated, and many postulations have been made.

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

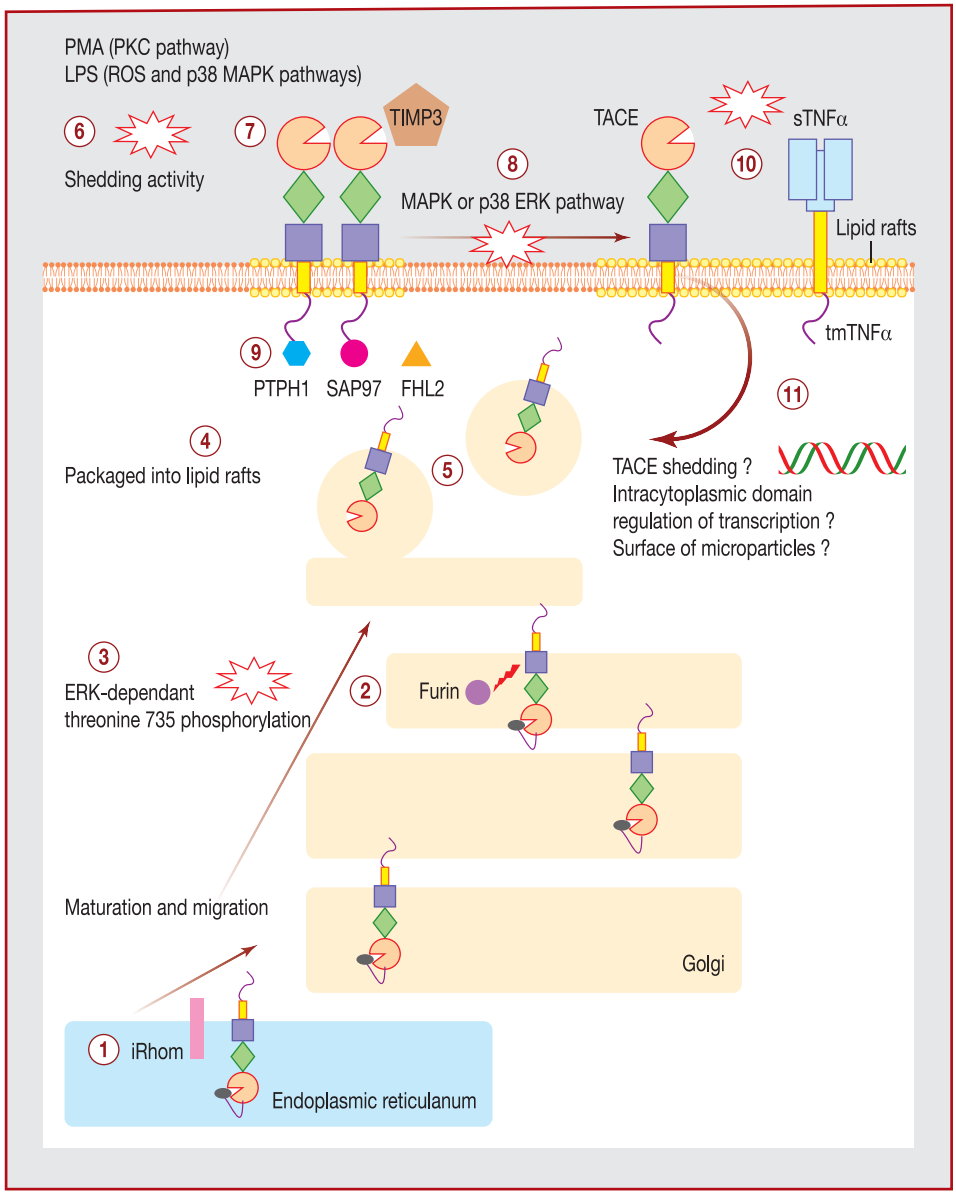


Figure 2. TACE activation and regulation. 1. Once synthesized, iRhom bind to TACE and promotes maturation, its exit from the endoplasmic reticulum and its migration to the Golgi. 2. TACE prodomain is cleaved by furin in the trans-Golgi network. 3. During maturation, ERK-dependent threonine 735 phosphorylation is necessary for TACE to reach the secretory pathway. 4. TACE is packaged into lipid rafts during its transport and maturation through the Golgi. 5. Most of the active form of TACE is localized in the cellular perinuclear region, with a small amount present in the plasma membrane. 6. TACE has an increased shedding rate when exposed to cell activators, such as phorbol esters, including PMA. Other activators include lipopolysaccharide, which is dependent on ROS and the p38 MAPK pathway. 7. TACE is present in dimers on the cell surface and binds to its inhibitor TIMP3. 8. Activation of the ERK or p38 MAPK pathway transforms TACE from a dimer structure into a monomer structure and releases it from TIMP3. 9. TACE is regulated by FHL2 and SAP97. 10. TACE cleaves transmembrane TNFα and releases soluble TNFα. ER: endoplasmic reticulum; ERK: extracellular signal-regulated kinases; FHL2: four and a half LIM domains 2; LPS: lipopolysaccharide; iRhom2: rhomboid family member 2; MAPK: mitogen-activated protein kinases; PKC: protein kinase C; PMA: phorbol myristate acetate; PTPH1: protein-tyrosine phosphatase; ROS: reactive oxygen species; SAP97: synapse-associated protein 97; sTNFα: soluble TNFα; TACE: tumour necrosis factor alpha converting enzyme; TIMP3: tissue inhibitor of metalloproteinases 3; tmTNFα: transmembrane TNFα; TNFα: tumour necrosis factor alpha.

ADAM10, another member of the ADAM family, is known to undergo regulated intramembrane proteolysis by presenilin after its ectodomain is shed by ADAM-9 or ADAM-15 [21]. In the intramembrane proteolysis process, a membrane protein typically undergoes two consecutive cleavages. The first results in the shedding of its ectodomain; the second one occurs within its transmembrane domain, resulting in secretion of a small peptide and the release of the intracellular domain into the cytosol. After intramembrane proteolysis, the cytoplasmic domain of ADAM10 can translocate to the nucleus to bind to gene loci undergoing transcription. Since ADAM10 is the closest relative of TACE, there is a high possibility that the cytoplasmic domain of TACE itself can undergo intramembrane

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

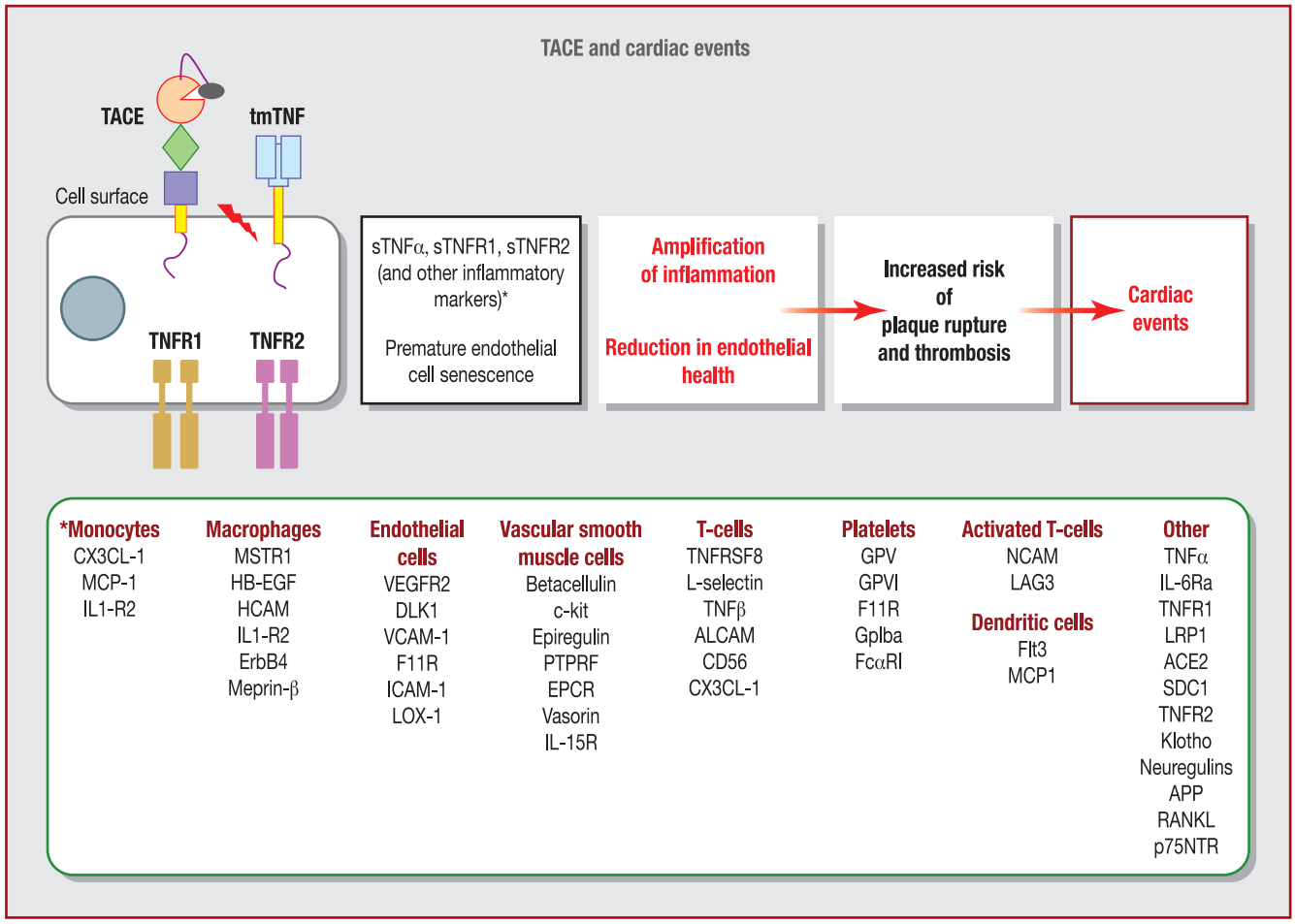


Figure 3. Possible role of TACE in cardiac events. This diagram shows the different proteins cleaved by TACE that are involved in chronic arterial wall inflammation occurring in atherosclerosis. As an example, TACE cleaves transmembrane TNFα, TNFR1 and TNFR2 releasing their soluble forms, sTNFα, sTNFR1 and sTNFR2, respectively. The transmembrane ectodomain shedding combined with a premature endothelial cell senescence due to TACE, leads to amplification of inflammation as well as reduction in endothelial health. This results in an increased risk of plaque rupture and thrombosis, and an overall increased risk of developing cardiovascular events. sTNFα: soluble tumour necrosis factor alfa; sTNFR1: soluble tumour necrosis factor alfa receptor 1; sTNFR2: soluble tumour necrosis factor alfa receptor 2; TACE: tumour necrosis factor alpha converting enzyme; tmTNFα: transmembrane TNFα; TNFα: tumour necrosis factor alfa; TNFR1: tumour necrosis factor alfa receptor 1; TNFR2: tumour necrosis factor alfa receptor 2.

proteolysis and participate in gene transcription regulation. A recent study reported that TACE can play a role in post-myocardial infarction recovery by regulating vascular endothelial growth factor receptor-2 transcription and angiogenesis in cardiomyocytes [22]. Whether TACE is internalized and downregulated after activation is still unclear. However, recent studies have reported that soluble TACE can be detected in the plasma [23,24], which is suggestive of a particular mechanism behind its own shedding, a process that remains unknown.

TACE and cardiovascular disease

TACE in heart diseases

Several studies have investigated the role of TACE in heart diseases. Investigators found that TACE expression was increased in endomyocardial tissues in myocarditis, as well as in the peripheral blood in advanced stages of heart failure. TACE was upregulated together with TNFα in myocarditis and negatively correlated with left ventricular systolic function [25]. Patients with advanced congestive heart failure also had an increased expression of TACE and TNFα compared to controls [26]. The role of TACE has been highlighted in aortic aneurysm [27] and in heart development [28]. Interestingly, Takayanagi et al. recently reported that TACE may be a novel therapeutic target for the prevention of hypertensive complications [29].

TACE and CAD

The underlying pathological process behind the development and progression of CAD is atherosclerosis, which results from an imbalance in lipid metabolism and a maladaptive immune response leading to chronic inflammation in the arterial wall. Major adverse cardiac events (MACE) often occur suddenly in patients with CAD after a revascularization strategy, resulting in high mortality and morbidity. These events include death, stroke, myocardial infarction, heart

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

failure and repeat coronary revascularization of the target lesion [30]. Many studies are currently investigating the association between different cardiovascular markers and MACE with a recent interest in multiple marker models versus a single marker model [31]. However, the investigation of TACE and associated proteins as a multiple marker model for MACE prediction has not yet been evaluated.

Clinical studies of TACE and associated proteins in CAD and AMI

Several studies have demonstrated that TACE plays a role in CAD initiation and progression to an acute coronary syndrome (ACS). Clinical studies have shown elevated plasma levels of TNFα in patients with AMI, suggesting that TNFα maturation, which relies on TACE, may activate systemic inflammation and contribute to plaque rupture [32]. Subsequent studies have found a positive association between TACE in AMI, as summarized in Table 1. Increased levels of gene expression of *TNFα* and *TACE* were found in circulating leucocytes of patients with myocardial infarction, obtained within 24 hours of onset [33]. The Killip-Kamball classification system is used in patients with an ACS to stratify their risk of mortality regarding the development of heart failure. Expression of *TACE* and *TNFα* was significantly higher in patients with Killip-Kamball class III or IV AMI than in those with class I or II AMI or in controls [34]. This finding demonstrates that higher levels of TACE-associated inflammation correlate with an increased risk of developing severe heart failure after an ACS. Shimoda et al. [35] reported that *TACE* gene expression levels in peripheral blood cells were higher in patients with AMI compared to healthy subjects, and particularly in those who had complications, such as malignant recurrent ventricular arrhythmia or pump failure. Both spontaneous and phorbol 12-myristate 13-acetate (PMA)-stimulated levels of *TACE* gene expression as well as TNFα gene and protein expression levels were found to be higher in patients with AMI compared to healthy subjects. Sustained increases in TACE and TNFα levels were reported 14 days after the onset of AMI and levels correlated positively with peak creatinine kinase levels.

Systemic gene expression levels of *TACE* and *TNFα* were documented to be higher in patients with AMI compared to patients with stable angina [36]. Interestingly, TACE levels were higher in local samples, near areas of ruptured coronary plaques, than in systemic samples obtained from patients with AMI. TACE and TNFα immunostaining showed that they were localized in infiltrating macrophages in ruptured coronary plaque/thrombus materials occluding the culprit coronary artery. In addition, increased levels of TACE in culprit coronary samples were the strongest independent predictor of adverse cardiac events (6 months after the onset of AMI) after adjustment for various clinical variables. The authors suggested that local expression of TACE in the culprit coronary artery leads to arterial remodelling and rupture or erosion of weakened coronary plaque, leading to the exacerbation of cardiac events [36].

Rizza et al. [37] recently measured TACE activity by evaluating the levels of its main four substrates (soluble VCAM-1, soluble ICAM, soluble IL6R and soluble TNFR1) in subjects with established vascular atherosclerosis who were followed for secondary MACE. They identified three homogeneous subgroups of patients, in terms of event risk, and an increased risk for incident events was observed among individuals with a high TACE score. Looking closely at TNFα receptors, soluble TNFR2 levels were found to be increased in heart failure, and other studies have shown higher circulating levels of soluble TNFR1 and soluble TNFR2 associated with nephropathy, cardiovascular events, and total mortality in type 2 diabetes [38]. Canault et al. demonstrated that TACE-containing microparticles of atherosclerotic plaques are partly of endothelial origin and that TACE on the surface of microparticles was still active [39]. A more recent study showed TACE activity in the plasma of patients with anti-neutrophil cytoplasmic autoantibodies vasculitis and indicated that TACE was also present on plasma microparticles derived mainly from platelets but also from endothelial cells. The authors reported that it is the active form of TACE that is detectable in plasma samples and that it is found on the surface of microparticles originating from platelets as well as endothelial cells [24].

Other studies have investigated the role of *TACE* polymorphisms in relation to cardiovascular disease. In the Athero*gene* study, TNFα, soluble TNFR1, and soluble TNFR2 concentrations were all significantly elevated in patients with future cardiovascular death. Moreover, individuals carrying the 747Leu allele in *TACE* displayed a borderline increased risk of future cardiovascular death. This study also suggests a role of TACE in the regulation of TNFα plasma levels and identified the *TACE* gene as a candidate for CAD risk [40].

TACE activation in CAD

The association of TACE with CAD is due first, to its role in shedding a variety of inflammatory molecules (Fig. 3) and second, to some of its activators that have been shown to be associated with atherosclerosis and pathophysiological functions in CAD.

TACE has an increased shedding rate when exposed to cell activators, such as phorbol esters (e.g. PMA) [41], the p38 MAPK pathway and lipopolysaccharide, which is dependent on reactive oxygen species stimulation [42]. Oxidative stress generating reactive oxygen species is known to be involved in the progression of atherosclerosis, disturbed blood flow and arterial wall remodelling [43]. When reactive oxygen species are generated, TACE activation is increased as a result local and systemic inflammation. Moreover, it is known that nitric oxide can activate TACE [44]. Nitric oxide is involved in the physiological regulation of blood flow and has pathophysiological functions in CAD [44]. On the other hand, C-reactive protein is also known to activate TACE and the release of soluble lectin-like oxidized low-density lipoprotein receptor-1, which plays an important role in the development and progression of atherosclerosis [45]. Therefore, TACE is not one inflammatory factor among many, but is a key enzyme. Its activators and subsequently shed proteins are essential mediators in the development and progression of CAD (Fig. 3).

TACE, associated proteins and endothelial dysfunction in CAD

TNFα is a major contributor to inflammatory processes in CAD. On cleavage of transmembrane TNFα by TACE,

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

Table 1 Summary of studies of TACE and associated proteins, and their clinical implications in cardiovascular disease.

Model/disease	Site of TACE measurement	Methods	Animals	Results summary	Reference
Heart disease: animal studies					
Mouse (atherosclerosis)	Atherosclerotic lesions	IHC FC ELISA	Five-week-old apoE−/− male mice examined at 5, 10, 15 and 25 weeks of age (14 in each group) and wild-type mice ($n=6$) were fed a high-fat diet	Strong expression of TACE in atherosclerosis-prone sites (aortic sinus and arch) Plasma levels of sTNFR1 and sTNFR2 correlated with atherosclerotic severity	Canault et al., 2006 [63]
Timp3−/− mouse	Heart tissue samples	qPCR WB	TIMP3-deficient mice ($n=11$) Age-matched wild-type littermates ($n=9$)	Loss of TIMP3 function triggered spontaneous left ventricular dilatation, cardiomyocyte hypertrophy, and contractile dysfunction at 21 months of age	Fedak et al., 2004 [33]
Rabbit	Sections from abdominal aorta Macrophages Tissues and cell lysate	IF qPCR WB	55 adult male New Zealand white rabbits and were then divided into 3 groups	TACE gene silencing reduced remodelling index and plaque burden and diminished the content of macrophages and lipids while increased that of smooth muscle cells and collagen in the aortic plaques The expression levels of TACE in unstable plaques were significantly higher than in stable plaques (42.6 ± 7.6 vs $25.2\pm6.5\%$, $P<0.01$)	Zhao et al., 2015 [64]
Heart disease: human studies					
Atherosclerosis	Surface of microparticles	FC IHC	25 patients (symptomatic, $n=10$; asymptomatic, $n=15$) undergoing carotid endarterectomy 3 controls	Human plaque microparticles carried catalytically active TACE and significantly enhanced the cell surface processing of the TACE substrates TNFα, TNFR1 and endothelial protein C receptor	Canault et al., 2007 [39]
Myocarditis	Endomyocardial biopsy tissues	qPCR IHC	14 patients 5 controls	TNFα and TACE expression was significantly greater in the myocarditis group than in the control group ($P<0.05$)	Satoh et al., 2000 [25]
CHF	PBMC	qPCR FC	46 patients 22 controls	TACE and TNFα gene expression, intracellular TACE and TNFα flow cytometry staining, and supernatant TNFα were higher in CHF patients than in controls ($P<0.001$)	Satoh et al., 2004 [26]
AAA	Aortic sample	IHC	39 patients 8 controls	TACE gene expression was increased in human AAA samples compared with normal aorta	Kaneko et al., 2011 [27]

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

Table 1 (*Continued*)

Model/disease	Site of TACE measurement	Methods	Animals	Results summary	Reference
AMI within 24 h of onset	Circulating leukocytes	qPCR	37 patients 8 controls	TACE. TNFα gene expression was higher in circulating leucocytes in AMI patients compared with controls ($P < 0.01$)	Akatsu et al., 2003 [34]
AMI (blood samples on day 1 and day 14 after onset of myocardial infarction)	PBMC	qPCR FC	41 patients 15 controls	TACE and TNFα gene expression levels were higher in AMI patients than in healthy controls ($P < 0.001$) Levels of TACE and TNFα decreased 14 days after the onset of AMI The percentage of TACE and TNFα cells with positive staining was higher in AMI patients compared with healthy controls ($P < 0.001$)	Shimoda et al., 2005 [35]
AMI	Local samples from the plaque site Systemic samples from the aorta Thrombus material	qPCR FC IHC	60 AMI patients 21 stable angina patients	TACE and TNFα gene expression and protein levels in both local and systemic samples obtained from AMI patients were higher than those levels in systemic samples obtained from stable angina patients ($P < 0.001$) In AMI patients, these levels were higher in local samples than in systemic samples ($P < 0.001$) By the 6-month follow-up study, local TACE levels remained the only significant independent predictor of adverse cardiac events in AMI	Satoh et al., 2008 [36]
Acute stage of atherosclerotic ischaemic stroke	Platelet-rich plasma and platelet total protein samples	WB	306 patients 230 controls	Plasma TACE levels in the atherosclerotic ischaemic stroke group were higher than those in the control group ($P = 0.70$, $P = 0.000$)	Ling et al., 2013 [65]
Human atherosclerotic plaques	Surgical waste from endarterectomy pieces	IHC	4 human atherosclerotic plaques	TACE gene expression was observed in human atherosclerotic plaques	Canault et al., 2007 [39]
Cardiac surgery with cardiopulmonary bypass	Plasma sample at three time points: before anaesthesia, at the beginning of sternal wiring, 48 h after the first sample	ELISA	25 patients	Analysis of TACE activity did not show significant differences between the different time points ($P = 0.40$), but a trend of the medians was apparent towards higher values postoperatively and after 48 hours	Erdoes et al., 2013 [66]

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

Table 1 (*Continued*)

Model/disease	Site of TACE measurement	Methods	Animals	Results summary	Reference
Other diseases: human studies					
MCI and AD	Cerebrospinal fluid and human plasma	FRET WB	64 patients with AD, 88 subjects with MCI, and 50 age-matched healthy controls	Plasma TACE protein levels did not differ significantly in the three study groups. However, plasma TACE activity in subjects with MCI and AD patients was significantly higher than that in HC ($P < 0.001$) TACE enzymatic activity may increase progressively over the clinical course of AD	Sun et al., 2014 [23]
AAV	Plasma samples	ELISA FRET FC IHC	PR3-AAV active ($n = 47$) PR3-AAV remission ($n = 45$) Disease control ($n = 14$)	TACE protein levels were significantly increased in plasma samples from patients with active PR3-AAV compared with samples from patients in remission or from other controls with renal nonvascular diseases Plasma TACE retained its specific proteolytic activity and was partly located on extracellular microparticles Transcript levels of TACE were increased in blood samples of patients with active AAV	Bertram et al., 2015 [24]
Clinical malaria	Plasma samples	ELISA	Village infections ($n = 6$), uncomplicated malaria ($n = 39$), severe malaria ($n = 123$), non-malaria hospitalized ($n = 32$), Acute severe malaria/convalescent ($n = 19$)	Plasma levels of TACE were increased in Tanzanian children hospitalized with a malaria infection compared with asymptomatic children, but were similar to children hospitalized with other infectious diseases Plasma levels of TACE decreased during recovery after an acute malaria episode	Petersen et al., 2016 [67]

AAA: abdominal aortic aneurysm; AAV: active proteinase-3 (PR3)-positive ANCA-associated vasculitis; AD: Alzheimer's disease; AMI: acute myocardial infarction; ANCA: antineutrophil cytoplasmic autoantibodies; CHF: congestive heart failure; ELISA: enzyme-linked immunosorbent assay; FC: flow cytometry; FRET: fluorescent resonance energy transfer; IF: immunofluorescence; IHC: immunohistochemistry assay; MIC: mild cognitive impairment; PBMC: peripheral blood mononuclear cells; qPCR: real time polymerase chain reaction; TIMP3: metalloproteinase inhibitor 3; TNFα: tumour necrosis factor alpha; TNFR1: TNFα receptor type 1; TNFR2: TNFα receptor type 2; sTNFR1: soluble TNFR1; sTNFR2: soluble TNFR2; WB: western blot.

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

upregulation of TNFα can promote premature endothelial cell senescence and can participate in the ageing process of coronary arteries [46]. Recently, endogenous transmembrane TNFα was shown to protect against premature senescence in endothelial colony forming cells [47]. This is supported by a murine study in which transgenic mice that only express an uncleavable version of transmembrane TNFα developed fewer inflammatory atherosclerotic plaques than the wild-type mice [48].

It has been also reported that TNFR1 activates multiple signalling pathways that have been linked to apoptosis, endothelial cell dysfunction and inflammation, whereas TNFR2 signalling has been proven to be beneficial to the cardiovascular system by activating angiogenic and survival pathways [49]. Transmembrane TNFα has a higher affinity for TNFR2, which confers a survival signal, mediating angiogenic and blood vessel repair activities [50]. This suggests that transmembrane TNFα cleavage by TACE can have a deleterious effect on the protective and repair function properties provided by transmembrane TNFα. The role of TACE in vascular dysfunction was also highlighted in patients and mice, where ageing and obesity cooperatively reduced caveolin-1 expression and increased vascular endothelial TACE activity and soluble TNFα release in adipose tissue. This was believed to contribute to the development of remote coronary microvascular dysfunction in older obese patients [46].

TACE inhibition

As TACE seems to be implicated in many physiological processes, it is important to consider its inhibition and the potential consequences. Interestingly, a patient with homozygous *TACE* deficiency was identified [52] who, despite repeated skin infections and episodes of bowel disease, led a relatively normal life, indicating that loss of *TACE* in humans might have less severe consequences than in rodents [53]. The most promising TACE inhibition (without any major physiological consequences) seems to lie in the inhibition of its regulators. iRhom1 and 2 are needed for TACE transport to the cell surface (Fig. 2) and it is well established that iRhom2 is predominantly expressed in immune cells, such as neutrophils and macrophages [54], whereas iRhom1 is mostly expressed on non-immune cells [55]. Therefore, it is tempting to speculate that inhibition of iRhom2 would lead to a selective deficiency of TACE in neutrophils and macrophages with no effects on keratinocytes or intestinal epithelial cells where, in these cell types, iRhom1 would compensate for the blockade of iRhom2. Recently, it was found that reducing the release of TNFα in cardiomyocytes by pharmacologically attenuating the phosphorylation of TACE, reduced TNFα shedding activity by TACE [56]. It was also proven possible to inhibit specifically TACE activity by using its natural inhibitory domain and consequently modulating TNFα secretion in cells [57]. Another strategy of inhibiting TACE could be an injection of its inhibitor TIMP3 in the heart, which has been shown to prevent heart failure post-myocardial infarction [58]. However, since TACE inhibition will reduce TNFα activity, it is important to consider the complex cardiac effects observed after TNFα inhibition as it is becoming increasingly clear that a minimum level of TNFα is important for the normal function of the heart [59,60].

Future work and conclusions

TACE plays a major role in controlling inflammatory processes and is involved in several chronic diseases. The number of known TACE substrates continues to increase, with mounting evidence that TACE is implicated in many cellular functions. Recent research indicates a particular role for TACE and the TNF family members in CAD and cardiovascular events, but none have really looked at this panel from a biomarker development point of view. Further investigations are, however, required to ascertain the exact role and mechanism of action of TACE in this disease area.

Future prospective studies in clinical cohorts at varying degrees of cardiovascular risk stratification are needed to fully assess TACE as a potential biomarker for CAD and MACE risk. This will be crucial for the future development of new personalized predictive tests and therapeutics that can improve patient clinical care pathways and prevent the high mortality rates associated with CAD.

Sources of funding

This research was supported by funds from Invest Northern Ireland, with additional funding obtained from the Health and social care research and development division, the university of Ulster and the Department of employment and learning in Northern Ireland.

Acknowledgements

All authors read and approved the final manuscript. This work was supported by grant of £11.5 M awarded to A.J.B. from the European union regional development fund, European union sustainable competitiveness programme for Northern Ireland; Northern Ireland public health agency (Health and social care research and development division) and the university of Ulster.

Disclosure of interest

The authors declare that they have no competing interest.

References

[1] Black RA, Rauch CT, Kozlosky CJ, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 1997;385:729—33.
[2] Schlöndorff J, Becherer JD, Blobel CP. Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). Biochem J 2000;347(Pt 1):131—8.
[3] Tellier E, Canault M, Rebsomen L, et al. The shedding activity of ADAM17 is sequestered in lipid rafts. Exp Cell Res 2006;312:3969—80.

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

[4] von Tresckow B, Kallen KJ, von Strandmann EP, et al. Depletion of cellular cholesterol and lipid rafts increases shedding of CD30. J. Immunol 2004;172:4324—31.
[5] Matthews V, Schuster B, Schütze S, et al. Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). J Biol Chem 2003;278:38829—39.
[6] Walev I, Tappe D, Gulbins E, Bhakdi S. Streptolysin O-permeabilized granulocytes shed L-selectin concomitantly with ceramide generation via neutral sphingomyelinase. J Leukoc Biol 2000;68:865—72.
[7] Tellier E, Canault M, Poggi M, et al. HDLs activate ADAM17-dependent shedding. J Cell Physiol 2008;214:687—93.
[8] Black RA. TIMP3 checks inflammation. Nat Genet 2004;36:934—5.
[9] Cardellini M, Menghini R, Luzi A, et al. Decreased IRS2 and TIMP3 expression in monocytes from offspring of type 2 diabetic patients is correlated with insulin resistance and increased intima-media thickness. Diabetes 2011;60:3265—70.
[10] Stöhr R, Kappel BA, Carnevale D, et al. TIMP3 interplays with apelin to regulate cardiovascular metabolism in hypercholesterolemic mice. Mol Metab 2015;4:741—52.
[11] Angoulvant D, Fazel S, Weisel RD, et al. Cell-based gene therapy modifies matrix remodeling after a myocardial infarction in tissue inhibitor of matrix metalloproteinase-3-deficient mice. J Thorac Cardiovasc Surg 2009;137:471—80.
[12] Tian H, Cimini M, Fedak PW, et al. TIMP-3 deficiency accelerates cardiac remodeling after myocardial infarction. J Mol Cell Cardiol 2007;43:733—43.
[13] Cesaro A, Abakar-Mahamat A, Brest P, et al. Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. Am J Physiol Gastrointest Liver Physiol 2009;296:G1332—43.
[14] Zheng Y, Schlondorff J, Blobel CP. Evidence for regulation of the tumor necrosis factor alpha-convertase (TACE) by protein-tyrosine phosphatase PTPH1. J Biol Chem 2002;277:42463—70.
[15] Stawikowska R, Cudic M, Giulianotti M, Houghten RA, Fields GB, Minond D. Activity of ADAM17 (a disintegrin and metalloprotease 17) is regulated by its noncatalytic domains and secondary structure of its substrates. J Biol Chem 2013;288:22871—9.
[16] Bergmeier W, Piffath CL, Cheng G, et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates GPIbalpha shedding from platelets in vitro and in vivo. Circ Res 2004;95:677—83.
[17] Arribas J, Esselens C. ADAM17 as a therapeutic target in multiple diseases. Curr Pharm Des 2009;15:2319—35.
[18] D'Alessio A, Esposito B, Giampietri C, Ziparo E, Pober JS, Filippini A. Plasma membrane microdomains regulate TACE-dependent TNFR1 shedding in human endothelial cells. J Cell Mol Med 2012;16:627—36.
[19] Odiete O, Hill MF, Sawyer DB. Neuregulin in cardiovascular development and disease. Circ Res 2012;111:1376—85.
[20] Wadugu B, Kühn B. The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. Am J Physiol Heart Circ Physiol 2012;302:H2139—47.
[21] Tousseyn T, Thathiah A, Jorissen E, et al. ADAM10, the rate-limiting protease of regulated intramembrane proteolysis of Notch and other proteins, is processed by ADAMS-9, ADAMS-15, and the gamma-secretase. J Biol Chem 2009;284:11738—47.
[22] Fan D, Takawale A, Shen M, et al. Cardiomyocyte A disintegrin and metalloproteinase 17 (ADAM17) is essential in post-myocardial infarction repair by regulating angiogenesis. Circ Heart Fail 2015;8:970—9.
[23] Sun Q, Hampel H, Blennow K, et al. Increased plasma TACE activity in subjects with mild cognitive impairment and patients with Alzheimer's disease. J Alzheimers Dis 2014;41:877—86.
[24] Bertram A, Lovric S, Engel A, et al. Circulating ADAM17 level reflects disease activity in proteinase-3 ANCA-associated vasculitis. J Am Soc Nephrol 2015;26:2860—70.
[25] Satoh M, Nakamura M, Satoh H, Saitoh H, Segawa I, Hiramori K. Expression of tumor necrosis factor-alpha — converting enzyme and tumor necrosis factor-alpha in human myocarditis. J Am Coll Cardiol 2000;36:1288—94.
[26] Satoh M, Iwasaka J, Nakamura M, Akatsu T, Shimoda Y, Hiramori K. Increased expression of tumor necrosis factor-alpha converting enzyme and tumor necrosis factor-alpha in peripheral blood mononuclear cells in patients with advanced congestive heart failure. Eur J Heart Fail 2004;6:869—75.
[27] Kaneko H, Anzai T, Horiuchi K, et al. Tumor necrosis factor-α converting enzyme is a key mediator of abdominal aortic aneurysm development. Atherosclerosis 2011;218:470—8.
[28] Shi W, Chen H, Sun J, et al. TACE is required for fetal murine cardiac development and modelling. Dev Biol 2003;261:371—80.
[29] Takayanagi T, Forrester SJ, Kawai T, et al. Vascular ADAM17 as a novel therapeutic target in mediating cardiovascular hypertrophy and perivascular fibrosis induced by angiotensin II novelty and significance. Hypertension 2016;68:949—55.
[30] Kip KE, Hollabaugh K, Marroquin OC, Williams DO. The problem with composite end points in cardiovascular studies: the story of major adverse cardiac events and percutaneous coronary intervention. J Am Coll Cardiol 2008;51:701—7.
[31] Wang TJ, Gona P, Larson MG, et al. Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med 2006;355:2631—9.
[32] Latini R, Bianchi M, Correale E, et al. Cytokines in acute myocardial infarction: selective increase in circulating tumor necrosis factor, its soluble receptor, and interleukin-1 receptor antagonist. J Cardiovasc Pharmacol 1994;23:1—6.
[33] Fedak PW, Smookler DS, Kassiri Z, et al. TIMP-3 deficiency leads to dilated cardiomyopathy. Circulation 2004;110:2401—9.
[34] Akatsu T, Nakamura M, Satoh M, Hiramori K. Increased mRNA expression of tumour necrosis factor-alpha and its converting enzyme in circulating leucocytes of patients with acute myocardial infarction. Clin Sci Lond 2003;105:39—44.
[35] Shimoda Y, Satoh M, Nakamura M, Akatsu T, Hiramori K. Activated tumour necrosis factor-alpha shedding process is associated with in-hospital complication in patients with acute myocardial infarction. Clin Sci Lond 2005;108:339—47.
[36] Satoh M, Ishikawa Y, Itoh T, Minami Y, Takahashi Y, Nakamura M. The expression of TNF-alpha converting enzyme at the site of ruptured plaques in patients with acute myocardial infarction. Eur J Clin Invest 2008;38:97—105.
[37] Rizza S, Copetti M, Cardellini M, et al. A score including ADAM17 substrates correlates to recurring cardiovascular event in subjects with atherosclerosis. Atherosclerosis 2015;239:459—64.
[38] Carlsson AC, Östgren CJ, Nystrom FH, et al. Association of soluble tumor necrosis factor receptors 1 and 2 with nephropathy, cardiovascular events, and total mortality in type 2 diabetes. Cardiovasc Diabetol 2016;15:40.
[39] Canault M, Leroyer AS, Peiretti F, et al. Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. Am J Pathol 2007;171:1713—23.
[40] Morange PE, Tregouet DA, Godefroy T, et al. Polymorphisms of the tumor necrosis factor-alpha (TNF) and the TNF-alpha converting enzyme (TACE/ADAM17) genes in relation to cardiovascular mortality: the AtheroGene study. J Mol Med (Berl) 2008;86:1153—61.
[41] Hooper NM, Karran EH, Turner AJ. Membrane protein secretases. Biochem J 1997;321(Pt 2):265—79.

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

[42] Scott AJ, O'Dea KP, O'Callaghan D, et al. Reactive oxygen species and p38 mitogen-activated protein kinase mediate tumor necrosis factor α-converting enzyme (TACE/ADAM-17) activation in primary human monocytes. J Biol Chem 2011;286:35466—76.
[43] He F, Zuo L. Redox roles of reactive oxygen species in cardiovascular diseases. Int J Mol Sci 2015;16:27770—80.
[44] Zhang Z, Kolls JK, Oliver P, et al. Activation of tumor necrosis factor-α-converting enzyme-mediated ectodomain shedding by nitric oxide. J Biol Chem 2000;275:15839—44.
[45] Zhao XQ, Zhang MW, Wang F, et al. CRP enhances soluble LOX-1 release from macrophages by activating TNF-α converting enzyme. J Lipid Res 2011;52:923—33.
[46] Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G. Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging. Physiol Genomics 2004;17:21—30.
[47] Green LA, Njoku V, Mund J, et al. Endogenous transmembrane TNF-alpha protects against premature senescence in endothelial colony forming cells. Circ Res 2016;118:1512—24.
[48] Canault M, Peiretti F, Mueller C, et al. Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus. Atherosclerosis 2004;172:211—8.
[49] Luo D, Luo Y, He Y, et al. Differential functions of tumor necrosis factor receptor 1 and 2 signaling in ischemia-mediated arteriogenesis and angiogenesis. Am J Pathol 2006;169:1886—98.
[50] Grell M, Becke FM, Wajant H, Männel DN, Scheurich P. TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. Eur J Immunol 1998;28:257—63.
[52] Blaydon DC, Biancheri P, Di WL, et al. Inflammatory skin and bowel disease linked to ADAM17 deletion. N Engl J Med 2011;365:1502—8.
[53] Peschon JJ, Slack JL, Reddy P, et al. An essential role for ectodomain shedding in mammalian development. Science 1998;282:1281—4.
[54] Adrain C, Zettl M, Christova Y, Taylor N, Freeman M. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. Science 2012;335(6065):225—8.
[55] Issuree PD, Maretzky T, McIlwain DR, et al. iRHOM2 is a critical pathogenic mediator of inflammatory arthritis. J Clin Invest 2013;123:928—32.
[56] Niu A, Wang B, Li Y-P. TNFα shedding in mechanically stressed cardiomyocytes is mediated by Src activation of TACE. J Cell Biochem 2015;116:559—65.
[57] Wong E, Cohen T, Romi E, et al. Harnessing the natural inhibitory domain to control TNFα converting enzyme (TACE) activity in vivo. Sci Rep 2016;6:35598.
[58] Martz L. Taking TIMP3 to heart. SciBX 2014;7:246.
[59] Cacciapaglia F, Menna P, Navarini L, Afeltra A, Salvatorelli E, Minotti G. Matters of the heart: the case of TNF alpha-targeting drugs. Mol Interv 2011;11:79—87.
[60] Balakumar P, Singh M. Anti-tumour necrosis factor-alpha therapy in heart failure: future directions. Basic Clin Pharmacol Toxicol 2006;99:391—7.
[63] Canault M, Peiretti F, Kopp F, et al. The TNF alpha converting enzyme (TACE/ADAM17) is expressed in the atherosclerotic lesions of apolipoprotein E-deficient mice: possible contribution to elevated plasma levels of soluble TNF alpha receptors. Atherosclerosis 2006;187:82—91.
[64] Zhao X, Kong J, Zhao Y, et al. Gene silencing of TACE enhances plaque stability and improves vascular remodeling in a rabbit model of atherosclerosis. Sci Rep 2015;5:17939.
[65] Ling JY, Shen L, Liu Q, et al. Changes in platelet GPIbα and ADAM17 during the acute stage of atherosclerotic ischemic stroke among Chinese. J Huazhong Univ Sci Technol Med Sci 2013;33:438—42.
[66] Erdoes G, Balmer ML, Slack E, et al. CD62L (L-Selectin) shedding for assessment of perioperative immune sensitivity in patients undergoing cardiac surgery with cardiopulmonary bypass. Plos One 2013;8:e53045.
[67] Petersen JE, Mkumbaye SI, Vaaben AV, et al. Plasma Ang2 and ADAM17 levels are elevated during clinical malaria; Ang2 level correlates with severity and expression of EPCR-binding PfEMP1. Sci Rep 2016;6:35950.

Please cite this article in press as: Chemaly M, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis (2017), https://doi.org/10.1016/j.acvd.2017.08.002

References

1. WHO | Global status report on noncommunicable diseases 2014 [Internet]. WHO. [cited 2018 May 10]. Available from: http://www.who.int/nmh/publications/ncd-status-report-2014/en/

2. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J. 2016 Nov 7;37(42):3232–45.

3. Newton JN, Briggs ADM, Murray CJL, Dicker D, Foreman KJ, Wang H, et al. Changes in health in England, with analysis by English regions and areas of deprivation, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. The Lancet. 2015 Dec 5;386(10010):2257–74.

4. Annual Report and Accounts 2015 [Internet]. [cited 2018 May 10]. Available from: https://www.bhf.org.uk/publications/about-bhf/annual-report-and-accounts-2015

5. Bhatnagar P, Wickramasinghe K, Wilkins E, Townsend N. Trends in the epidemiology of cardiovascular disease in the UK. Heart. 2016 Jul 26;heartjnl-2016-309573.

6. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011 Nov 7;17(11):1410–22.

7. Tabas I, García-Cardeña G, Owens GK. Recent insights into the cellular biology of atherosclerosis. J Cell Biol. 2015 Apr 13;209(1):13–22.

8. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. Arterioscler Thromb Vasc Biol. 1995 May;15(5):551–61.

9. Packard RRS, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem. 2008 Jan;54(1):24–38.

10. Falk E, Nakano M, Bentzon JF, Finn AV, Virmani R. Update on acute coronary syndromes: the pathologists' view. Eur Heart J. 2013 Mar;34(10):719–28.

11. Virmani R, Burke AP, Kolodgie FD, Farb A. Vulnerable plaque: the pathology of unstable coronary lesions. J Intervent Cardiol. 2002 Dec;15(6):439–46.

12. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011 Mar;12(3):204–12.

13. Stefanadis C, Antoniou C-K, Tsiachris D, Pietri P. Coronary Atherosclerotic Vulnerable Plaque: Current Perspectives. J Am Heart Assoc. 2017 Mar 1;6(3):e005543.

14. Capodanno D, Di Salvo ME, Cincotta G, Miano M, Tamburino C, Tamburino C. Usefulness of the SYNTAX score for predicting clinical outcome after percutaneous coronary intervention of unprotected left main coronary artery disease. Circ Cardiovasc Interv. 2009 Aug;2(4):302–8.

15. Kip KE, Hollabaugh K, Marroquin OC, Williams DO. The problem with composite end points in cardiovascular studies: the story of major adverse cardiac events and percutaneous coronary intervention. J Am Coll Cardiol. 2008 Feb 19;51(7):701–7.

16. Wallentin L, Lindholm D, Siegbahn A, Wernroth L, Becker RC, Cannon CP, et al. Biomarkers in relation to the effects of ticagrelor in comparison with clopidogrel in non-ST-elevation acute coronary syndrome patients managed with or without in-hospital

revascularization: a substudy from the Prospective Randomized Platelet Inhibition and Patient Outcomes (PLATO) trial. Circulation. 2014 Jan 21;129(3):293–303.

17. Mega JL, Braunwald E, Wiviott SD, Bassand J-P, Bhatt DL, Bode C, et al. Rivaroxaban in Patients with a Recent Acute Coronary Syndrome. N Engl J Med. 2012 Jan 5;366(1):9–19.

18. Jernberg T, Hasvold P, Henriksson M, Hjelm H, Thuresson M, Janzon M. Cardiovascular risk in post-myocardial infarction patients: nationwide real world data demonstrate the importance of a long-term perspective. Eur Heart J. 2015 May 14;36(19):1163–70.

19. Glaser R, Selzer F, Faxon DP, Laskey WK, Cohen HA, Slater J, et al. Clinical Progression of Incidental, Asymptomatic Lesions Discovered During Culprit Vessel Coronary Intervention. Circulation. 2005 Jan 18;111(2):143–9.

20. Lewis SJ, Moye LA, Sacks FM, Johnstone DE, Timmis G, Mitchell J, et al. Effect of pravastatin on cardiovascular events in older patients with myocardial infarction and cholesterol levels in the average range. Results of the Cholesterol and Recurrent Events (CARE) trial. Ann Intern Med. 1998 Nov 1;129(9):681–9.

21. Stevens SL, Wood S, Koshiaris C, Law K, Glasziou P, Stevens RJ, et al. Blood pressure variability and cardiovascular disease: systematic review and meta-analysis. BMJ. 2016 Aug 9;354:i4098.

22. Kargarfard M, Rouzbehani R, Basati F. Effects of Exercise Rehabilitation on Blood Pressure of Patients after Myocardial Infarction. Int J Prev Med. 2010;1(2):124–30.

23. Kapur A, De Palma R. Mortality after myocardial infarction in patients with diabetes mellitus. Heart. 2007 Dec;93(12):1504–6.

24. Stefanadis C, Dernellis J, Tsiamis E, Stratos C, Diamantopoulos L, Michaelides A, et al. Aortic stiffness as a risk factor for recurrent acute coronary events in patients with ischaemic heart disease. Eur Heart J. 2000 Mar 1;21(5):390–6.

25. Akkus O, Sahin DY, Bozkurt A, Nas K, Ozcan KS, Illyés M, et al. Evaluation of Arterial Stiffness for Predicting Future Cardiovascular Events in Patients with ST Segment Elevation and Non-ST Segment Elevation Myocardial Infarction. Sci World J [Internet]. 2013 Oct 30 [cited 2017 Aug 16];2013. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3833000/

26. Sethi S, Rivera O, Oliveros R, Chilton R. Aortic stiffness: pathophysiology, clinical implications, and approach to treatment. Integr Blood Press Control. 2014 May 23;7:29–34.

27. Hajj-Ali R, Zareba W, Ezzeddine R, Moss AJ. Relation of the leukocyte count to recurrent cardiac events in stable patients after acute myocardial infarction. Am J Cardiol. 2001 Dec 1;88(11):1221–4.

28. Mylotte D, Foley D, Kenny D. Platelet function testing: methods of assessment and clinical utility. Cardiovasc Hematol Agents Med Chem. 2011 Jan;9(1):14–24.

29. Park J-H, Tahk S-J, Bae SH. Depression and anxiety as predictors of recurrent cardiac events 12 months after percutaneous coronary interventions. J Cardiovasc Nurs. 2015 Aug;30(4):351–9.

30. Lloyd-Jones DM. Cardiovascular risk prediction: basic concepts, current status, and future directions. Circulation. 2010 Apr 20;121(15):1768–77.

31. Califf RM, Armstrong PW, Carver JR, D'Agostino RB, Strauss WE. 27th Bethesda Conference: matching the intensity of risk factor management with the hazard for coronary disease events. Task Force 5. Stratification of patients into high, medium and low risk subgroups for purposes of risk factor management. J Am Coll Cardiol. 1996 Apr;27(5):1007–19.

32. Wilson PWF, D'Agostino R, Bhatt DL, Eagle K, Pencina MJ, Smith SC, et al. An international model to predict recurrent cardiovascular disease. Am J Med. 2012 Jul;125(7):695–703.e1.

33. Hribal ML. A new score to predict recurrent cardiovascular events? Atherosclerosis. 2015 May 1;240(1):1–2.

34. Strong JP, Malcom GT, McMahan CA, Tracy RE, Iii WPN, Herderick EE, et al. Prevalence and Extent of Atherosclerosis in Adolescents and Young Adults: Implications for Prevention From the Pathobiological Determinants of Atherosclerosis in Youth Study. JAMA. 1999 Feb 24;281(8):727–35.

35. van Loon JE, de Maat MPM, Deckers JW, van Domburg RT, Leebeek FWG. Prognostic markers in young patients with premature coronary heart disease. Atherosclerosis. 2012 Sep 1;224(1):213–7.

36. Mendis S, Thygesen K, Kuulasmaa K, Giampaoli S, Mähönen M, Ngu Blackett K, et al. World Health Organization definition of myocardial infarction: 2008–09 revision. Int J Epidemiol. 2011 Feb 1;40(1):139–46.

37. Steg PG, James SK, Atar D, Badano LP, Lundqvist CB, Borger MA, et al. ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevationThe Task Force on the management of ST-segment elevation acute myocardial infarction of the European Society of Cardiology (ESC). Eur Heart J. 2012 Oct 1;33(20):2569–619.

38. Roffi M, Patrono C, Collet J-P, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevationTask Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J. 2016 Jan 14;37(3):267–315.

39. Ren L, Ye H, Wang P, Cui Y, Cao S, Lv S. Comparison of long-term mortality of acute ST-segment elevation myocardial infarction and non-ST-segment elevation acute coronary syndrome patients after percutaneous coronary intervention. Int J Clin Exp Med. 2014 Dec 15;7(12):5588–92.

40. Kumar A, Cannon CP. Acute Coronary Syndromes: Diagnosis and Management, Part I. Mayo Clin Proc. 2009 Oct;84(10):917–38.

41. Montalescot G. STEMI and NSTEMI are two distinct pathophysiological entities: reply. Eur Heart J. 2007 Nov 1;28(21):2685–6.

42. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Executive summary: heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation. 2012 Jan 3;125(1):188–97.

43. Scharnhorst V, Krasznai K, van't Veer M, Michels R. Rapid Detection of Myocardial Infarction With a Sensitive Troponin Test. Am J Clin Pathol. 2011 Mar 1;135(3):424–8.

44. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, et al. Third universal definition of myocardial infarction. J Am Coll Cardiol. 2012 Oct 16;60(16):1581–98.

45. Braunwald E, Morrow DA. Unstable angina: is it time for a requiem? Circulation. 2013 Jun 18;127(24):2452–7.

46. Mueller C. Biomarkers and acute coronary syndromes: an update. Eur Heart J. 2014 Mar;35(9):552–6.

47. Reichlin T, Twerenbold R, Maushart C, Reiter M, Moehring B, Schaub N, et al. Risk stratification in patients with unstable angina using absolute serial changes of 3 high-sensitive troponin assays. Am Heart J. 2013 Mar;165(3):371–378.e3.

48. Bodor GS, Porterfield D, Voss EM, Smith S, Apple FS. Cardiac troponin-I is not expressed in fetal and healthy or diseased adult human skeletal muscle tissue. Clin Chem. 1995 Dec;41(12 Pt 1):1710–5.

49. Shave R, Dawson E, Whyte G, George K, Ball D, Collinson P, et al. The cardiospecificity of the third-generation cTnT assay after exercise-induced muscle damage. Med Sci Sports Exerc. 2002 Apr;34(4):651–4.

50. Daubert MA, Jeremias A. The utility of troponin measurement to detect myocardial infarction: review of the current findings. Vasc Health Risk Manag. 2010;6:691–9.

51. Tate JR. Troponin revisited 2008: assay performance. Clin Chem Lab Med. 2008;46(11):1489–500.

52. Alpert JS, Thygesen K, Antman E, Bassand JP. Myocardial infarction redefined--a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. J Am Coll Cardiol. 2000 Sep;36(3):959–69.

53. Sharma S, Jackson PG, Makan J. Cardiac troponins. J Clin Pathol. 2004 Oct;57(10):1025–6.

54. Adams JE, Abendschein DR, Jaffe AS. Biochemical markers of myocardial injury. Is MB creatine kinase the choice for the 1990s? Circulation. 1993 Aug;88(2):750–63.

55. O'Donnell CJ, Elosua R, O'Donnell CJ, Elosua R. Cardiovascular Risk Factors. Insights From Framingham Heart Study. Rev Esp Cardiol. 2008 Jan 3;61(03):299–310.

56. Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation. 1983 May 1;67(5):968–77.

57. Stamler J, Vaccaro O, Neaton JD, Wentworth D, Group TMRFITR. Diabetes, Other Risk Factors, and 12-Yr Cardiovascular Mortality for Men Screened in the Multiple Risk Factor Intervention Trial. Diabetes Care. 1993 Feb 1;16(2):434–44.

58. Ludwig DS, Pereira MA, Kroenke CH, Hilner JE, Horn LV, Slattery ML, et al. Dietary Fiber, Weight Gain, and Cardiovascular Disease Risk Factors in Young Adults. JAMA. 1999 Oct 27;282(16):1539–46.

59. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, et al. Kidney Disease as a Risk Factor for Development of Cardiovascular Disease: A Statement From the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation. 2003 Oct 28;108(17):2154–69.

60. Mancia G, Fagard R, Narkiewicz K, Redón J, Zanchetti A, Böhm M, et al. 2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J Hypertens. 2013 Jul;31(7):1281–357.

61. Catapano AL, Graham I, De Backer G, Wiklund O, Chapman MJ, Drexel H, et al. 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias. Eur Heart J. 2016 Oct 14;37(39):2999–3058.

62. Luft FC. Renal disease as a risk factor for cardiovascular disease. Basic Res Cardiol. 2000;95 Suppl 1:I72-76.

63. Reiner Ž, Catapano AL, De Backer G, Graham I, Taskinen M-R, Wiklund O, et al. ESC/EAS Guidelines for the management of dyslipidaemiasThe Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Eur Heart J. 2011 Jul 1;32(14):1769–818.

64. Alpert PT. New and emerging theories of cardiovascular disease: infection and elevated iron. Biol Res Nurs. 2004 Jul;6(1):3–10.

65. Emdin CA, Wong CX, Hsiao AJ, Altman DG, Peters SA, Woodward M, et al. Atrial fibrillation as risk factor for cardiovascular disease and death in women compared with men: systematic review and meta-analysis of cohort studies. BMJ. 2016 Jan 19;352:h7013.

66. Linstra KM, Wermer MJH, Terwindt GM. [Women with migraine have an increased risk of cardiovascular disease; what are the consequences for the clinical management of young patients?]. Ned Tijdschr Geneeskd. 2017;161(0):D1143.

67. Eriksson JK, Jacobsson L, Bengtsson K, Askling J. Is ankylosing spondylitis a risk factor for cardiovascular disease, and how do these risks compare with those in rheumatoid arthritis? Ann Rheum Dis. 2017 Feb 1;76(2):364–70.

68. Shalen EF, Nah G, Vittinghoff E, Tseng ZH, Marcus GM, Albert MA, et al. Abstract P333: Homelessness is an Independent Risk Factor for Cardiovascular Disease Hospital Readmission in the California Health Care Utilization Project. Circulation. 2017 Mar 7;135(Suppl 1):AP333-AP333.

69. Allgulander C. Anxiety as a risk factor in cardiovascular disease. Curr Opin Psychiatry. 2016 Jan;29(1):13–7.

70. Foley S, Carpenter K, Schwab P, Schaffner C, Hartos J. Is Depression a Risk Factor for Cardiovascular Disease in Middle-Aged Women? Res Apprec Day [Internet]. 2017 Mar 14; Available from: http://digitalcommons.hsc.unt.edu/rad/RAD17/Cardiovascular/9

71. Valtorta NK, Kanaan M, Gilbody S, Ronzi S, Hanratty B. Loneliness and social isolation as risk factors for coronary heart disease and stroke: systematic review and meta-analysis of longitudinal observational studies. Heart. 2016 Jul 1;102(13):1009–16.

72. Morris CJ, Purvis TE, Hu K, Scheer FAJL. Circadian misalignment increases cardiovascular disease risk factors in humans. Proc Natl Acad Sci. 2016 Mar 8;113(10):E1402–11.

73. Ma W, Rimm EB, Rexrode KM, Qi L. Abstract P080: Associations of Bowel Movement Frequency with Risk of Coronary Heart Disease and Mortality Among Us Women. Circulation. 2016 Mar 1;133(Suppl 1):AP080-AP080.

74. Liou S-H, Tsai S-S, Shih T-S, Lin M-H, Wu W-T. P063 Sleep apnea as an independent risk factor for cardiovascular disease among professional drivers: evidence from the taiwan bus driver cohort study (TBDCS). Occup Env Med. 2016 Sep 1;73(Suppl 1):A142–A142.

75. Stanhope KL, Medici V, Bremer AA, Lee V, Lam HD, Nunez MV, et al. A dose-response study of consuming high-fructose corn syrup–sweetened beverages on lipid/lipoprotein risk factors for cardiovascular disease in young adults. Am J Clin Nutr. 2015 Jun 1;101(6):1144–54.

76. Moran AE, Forouzanfar MH, Roth GA, Mensah GA, Ezzati M, Murray CJL, et al. Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: the Global Burden of Disease 2010 study. Circulation. 2014 Apr 8;129(14):1483–92.

77. Mason H, Shoaibi A, Ghandour R, O'Flaherty M, Capewell S, Khatib R, et al. A Cost Effectiveness Analysis of Salt Reduction Policies to Reduce Coronary Heart Disease in Four Eastern Mediterranean Countries. PLOS ONE. 2014 Jan 7;9(1):e84445.

78. O'Keeffe C, Kabir Z, O'Flaherty M, Walton J, Capewell S, Perry IJ. Modelling the impact of specific food policy options on coronary heart disease and stroke deaths in Ireland. BMJ Open. 2013 Jul 1;3(7):e002837.

79. Liu K, Daviglus ML, Loria CM, Colangelo LA, Spring B, Moller AC, et al. Healthy lifestyle through young adulthood and the presence of low cardiovascular disease risk profile in middle age: the Coronary Artery Risk Development in (Young) Adults (CARDIA) study. Circulation. 2012 Feb 28;125(8):996–1004.

80. Ezzati M, Organització Mundial de la Salut. Comparative quantification of health risks: global and regional burden of disease attributable to selected major risk factors. Geneva: World Health Organization; 2004.

81. Stewart J, Manmathan G, Wilkinson P. Primary prevention of cardiovascular disease: A review of contemporary guidance and literature. JRSM Cardiovasc Dis. 2017 Jan 1;6:2048004016687211.

82. Vandvik PO, Lincoff AM, Gore JM, Gutterman DD, Sonnenberg FA, Alonso-Coello P, et al. Primary and Secondary Prevention of Cardiovascular Disease. Chest. 2012 Feb;141(2 Suppl):e637S–e668S.

83. Conroy RM, Pyörälä K, Fitzgerald AP, Sans S, Menotti A, De Backer G, et al. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. Eur Heart J. 2003 Jun;24(11):987–1003.

84. Cooney MT, Dudina A, De Bacquer D, Wilhelmsen L, Sans S, Menotti A, et al. HDL cholesterol protects against cardiovascular disease in both genders, at all ages and at all levels of risk. Atherosclerosis. 2009 Oct;206(2):611–6.

85. Cooney MT, Dudina A, De Bacquer D, Fitzgerald A, Conroy R, Sans S, et al. How much does HDL cholesterol add to risk estimation? A report from the SCORE Investigators. Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol. 2009 Jun;16(3):304–14.

86. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. JAMA. 2007 Jul 18;298(3):309–16.

87. Versteylen MO, Joosen IA, Shaw LJ, Narula J, Hofstra L. Comparison of Framingham, PROCAM, SCORE, and Diamond Forrester to predict coronary atherosclerosis and cardiovascular events. J Nucl Cardiol. 2011 Oct;18(5):904–11.

88. Gyberg V, Bacquer DD, Kotseva K, Backer GD, Schnell O, Tuomilehto J, et al. Time-saving screening for diabetes in patients with coronary artery disease: a report from EUROASPIRE IV. BMJ Open. 2016 Dec 1;6(12):e013835.

89. Mayor S. New scoring system predicts cardiovascular disease risk worldwide. BMJ. 2015 Mar 26;350:h1670.

90. D'Agostino RB, Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. Circulation. 2008 Feb 12;117(6):743–53.

91. Woodward M, Brindle P, Tunstall-Pedoe H, SIGN group on risk estimation. Adding social deprivation and family history to cardiovascular risk assessment: the ASSIGN score from the Scottish Heart Health Extended Cohort (SHHEC). Heart Br Card Soc. 2007 Feb;93(2):172–6.

92. Hippisley-Cox J, Coupland C, Vinogradova Y, Robson J, May M, Brindle P. Derivation and validation of QRISK, a new cardiovascular disease risk score for the United Kingdom: prospective open cohort study. BMJ. 2007 Jul 19;335(7611):136.

93. Hippisley-Cox J, Coupland C, Vinogradova Y, Robson J, Minhas R, Sheikh A, et al. Predicting cardiovascular risk in England and Wales: prospective derivation and validation of QRISK2. BMJ. 2008 Jun 26;336(7659):1475–82.

94. Assmann G, Cullen P, Schulte H. Simple scoring scheme for calculating the risk of acute coronary events based on the 10-year follow-up of the prospective cardiovascular Münster (PROCAM) study. Circulation. 2002 Jan 22;105(3):310–5.

95. Cooney MT, Dudina AL, Graham IM. Value and Limitations of Existing Scores for the Assessment of Cardiovascular Risk. J Am Coll Cardiol. 2009 Sep 29;54(14):1209–27.

96. D'Agostino RB, Grundy S, Sullivan LM, Wilson P, CHD Risk Prediction Group. Validation of the Framingham coronary heart disease prediction scores: results of a multiple ethnic groups investigation. JAMA. 2001 Jul 11;286(2):180–7.

97. Cook NR. Use and misuse of the receiver operating characteristic curve in risk prediction. Circulation. 2007 Feb 20;115(7):928–35.

98. Jackson R. Cardiovascular risk prediction: are we there yet? Heart Br Card Soc. 2008 Jan;94(1):1–3.

99. Moons KGM, Kengne AP, Woodward M, Royston P, Vergouwe Y, Altman DG, et al. Risk prediction models: I. Development, internal validation, and assessing the incremental value of a new (bio)marker. Heart Br Card Soc. 2012 May;98(9):683–90.

100. Cook NR, Paynter NP, Eaton CB, Manson JE, Martin LW, Robinson JG, et al. Comparison of the Framingham and Reynolds Risk scores for global cardiovascular risk prediction in the multiethnic Women's Health Initiative. Circulation. 2012 Apr 10;125(14):1748–56, S1-11.

101. Everett BM, Brooks MM, Vlachos HEA, Chaitman BR, Frye RL, Bhatt DL. Troponin and Cardiac Events in Stable Ischemic Heart Disease and Diabetes. N Engl J Med. 2015 Aug 13;373(7):610–20.

102. Nadir MA, Rekhraj S, Wei L, Lim TK, Davidson J, MacDonald TM, et al. Improving the primary prevention of cardiovascular events by using biomarkers to identify individuals with silent heart disease. J Am Coll Cardiol. 2012 Sep 11;60(11):960–8.

103. Emerging Risk Factors Collaboration, Di Angelantonio E, Gao P, Khan H, Butterworth AS, Wormser D, et al. Glycated hemoglobin measurement and prediction of cardiovascular disease. JAMA. 2014 Mar 26;311(12):1225–33.

104. Berry JD, Lloyd-Jones DM, Garside DB, Greenland P. FRAMINGHAM RISK SCORE AND PREDICTION OF CORONARY HEART DISEASE DEATH IN YOUNG MEN. Am Heart J. 2007 Jul;154(1):80–6.

105. Aragam KG, Tamhane UU, Kline-Rogers E, Li J, Fox KAA, Goodman SG, et al. Does simplicity compromise accuracy in ACS risk prediction? A retrospective analysis of the TIMI and GRACE risk scores. PloS One. 2009 Nov 23;4(11):e7947.

106. de Araújo Gonçalves P, Ferreira J, Aguiar C, Seabra-Gomes R. TIMI, PURSUIT, and GRACE risk scores: sustained prognostic value and interaction with revascularization in NSTE-ACS. Eur Heart J. 2005 May;26(9):865–72.

107. TRACE-CORE Investigators, Huang W, FitzGerald G, Goldberg R, Gore J, McManus R, et al. Performance of the GRACE Risk Score 2.0 Simplified Algorithm for Predicting 1-Year Death After Hospitalization for an Acute Coronary Syndrome in a Contemporary Multiracial Cohort. GRACE Publ [Internet]. 2016 Oct 15; Available from: http://escholarship.umassmed.edu/cor_grace/125

108. Fox KAA, Fitzgerald G, Puymirat E, Huang W, Carruthers K, Simon T, et al. Should patients with acute coronary disease be stratified for management according to their risk? Derivation, external validation and outcomes using the updated GRACE risk score. BMJ Open. 2014 Feb 21;4(2):e004425.

109. Backus B., Six A., Kelder J., Gibler W., Moll F., Doevendans P. Risk Scores for Patients with Chest Pain: Evaluation in the Emergency Department. Curr Cardiol Rev. 2011 Feb;7(1):2–8.

110. Manfrini O, Bugiardini R. Barriers to clinical risk scores adoptionThe opinions expressed in this article are not necessarily those of the Editors of the European Heart Journal or of the European Society of Cardiology. Eur Heart J. 2007 May 1;28(9):1045–6.

111. Fox N, Growdon JH. Biomarkers and Surrogates. NeuroRx. 2004 Apr;1(2):181.

112. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001 Mar;69(3):89–95.

113. Manolio T. Novel Risk Markers and Clinical Practice. N Engl J Med. 2003 Oct 23;349(17):1587–9.

114. Šimundić A-M. Measures of Diagnostic Accuracy: Basic Definitions. EJIFCC. 2009 Jan 20;19(4):203–11.

115. Armbruster DA, Pry T. Limit of Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev. 2008 Aug;29(Suppl 1):S49–52.

116. LaBaer J. So, you want to look for biomarkers (introduction to the special biomarkers issue). J Proteome Res. 2005 Aug;4(4):1053–9.

117. Morrow DA, de Lemos JA, Sabatine MS, Antman EM. The search for a biomarker of cardiac ischemia. Clin Chem. 2003 Apr;49(4):537–9.

118. Hlatky MA. Exercise Testing to Predict Outcome in Patients with Angina. J Gen Intern Med. 1999 Jan;14(1):63–5.

119. Mushlin AI, Kern LM, Paris M, Lambert DR, Williams G. The value of diagnostic information to patients with chest pain suggestive of coronary artery disease. Med Decis Mak Int J Soc Med Decis Mak. 2005 Apr;25(2):149–57.

120. Zethelius B, Berglund L, Sundström J, Ingelsson E, Basu S, Larsson A, et al. Use of Multiple Biomarkers to Improve the Prediction of Death from Cardiovascular Causes. N Engl J Med. 2008 May 15;358(20):2107–16.

121. Jickling GC, Sharp FR. Biomarker Panels in Ischemic Stroke. Stroke. 2015 Mar 1;46(3):915–20.

122. Yin X, Subramanian S, Hwang S-J, O'Donnell CJ, Fox CS, Courchesne P, et al. Protein Biomarkers of New-Onset Cardiovascular Disease: Prospective Study From the Systems Approach to Biomarker Research in Cardiovascular Disease Initiative. Arterioscler Thromb Vasc Biol. 2014 Jan 1;ATVBAHA.113.302918.

123. Halim SA, Neely ML, Pieper KS, Shah SH, Kraus WE, Hauser ER, et al. Simultaneous Consideration of Multiple Candidate Protein Biomarkers for Long-Term Risk for Cardiovascular Events. Circ Cardiovasc Genet. 2014 Nov 24;CIRCGENETICS.113.000490.

124. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the Areas under Two or More Correlated Receiver Operating Characteristic Curves: A Nonparametric Approach. Biometrics. 1988;44(3):837–45.

125. Fortmann SP, Ford E, Criqui MH, Folsom AR, Harris TB, Hong Y, et al. CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: report from the population science discussion group. Circulation. 2004 Dec 21;110(25):e554-559.

126. Solberg HE. Approved recommendation (1986) on the theory of reference values. Part 1. The concept of reference values. Clin Chim Acta. 1987 May 29;165(1):111–8.

127. PetitClerc C, Solberg HE. Approved recommendation (1987) on the theory of reference values. Part 2. Selection of individuals for the production of reference values. Clin Chim Acta. 1987 Dec 1;170(2):S1–11.

128. Solberg HE. Approved recommendation (1987) on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. Clin Chim Acta. 1987 Dec 1;170(2):S13–32.

129. Sunderman FW. Current concepts of “normal values,” “reference values,” and “discrimination values,” in clinical chemistry. Clin Chem. 1975 Dec;21(13):1873–7.

130. Shapiro BP, Chen HH, Burnett JC, Redfield MM. Use of plasma brain natriuretic peptide concentration to aid in the diagnosis of heart failure. Mayo Clin Proc. 2003 Apr;78(4):481–6.

131. Sebastián-Gámbaro MA, Lirón-Hernández FJ, Fuentes-Arderiu X. Intra- and inter-individual biological variability data bank. Eur J Clin Chem Clin Biochem J Forum Eur Clin Chem Soc. 1997 Nov;35(11):845–52.

132. Guder WG. Preanalytical factors and their influence on analytical quality specifications. Scand J Clin Lab Invest. 1999 Jan 1;59(7):545–9.

133. Fraser CG, Petersen PH. Analytical performance characteristics should be judged against objective quality specifications. Clin Chem. 1999 Mar;45(3):321–3.

134. Pompanon F, Bonin A, Bellemain E, Taberlet P. Genotyping errors: causes, consequences and solutions. Nat Rev Genet. 2005 Nov;6(11):847–59.

135. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet. 2001 Dec;29(4):365–71.

136. Price CP. Point of care testing. BMJ. 2001 May 26;322(7297):1285–8.

137. Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. Circulation. 2006 May 16;113(19):2335–62.

138. de Lemos JA, Hennekens CH, Ridker PM. Plasma concentration of soluble vascular cell adhesion molecule-1 and subsequent cardiovascular risk. J Am Coll Cardiol. 2000 Aug;36(2):423–6.

139. Cybulsky MI, Gimbrone MA. Endothelial Expression of a Mononuclear Leukocyte Adhesion Molecule during Atherogenesis. Sci Wash. 1991 Feb 15;251(4995):788.

140. Li H, Cybulsky MI, Gimbrone MA, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb J Vasc Biol. 1993 Feb;13(2):197–204.

141. Li H, Cybulsky MI, Gimbrone MA, Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. Am J Pathol. 1993 Dec;143(6):1551–9.

142. Gerszten RE, Luscinskas FW, Ding HT, Dichek DA, Stoolman LM, Gimbrone MA, et al. Adhesion of memory lymphocytes to vascular cell adhesion molecule-1-transduced

human vascular endothelial cells under simulated physiological flow conditions in vitro. Circ Res. 1996 Dec;79(6):1205–15.

143. Gerszten RE, Lim YC, Ding HT, Snapp K, Kansas G, Dichek DA, et al. Adhesion of monocytes to vascular cell adhesion molecule-1-transduced human endothelial cells: implications for atherogenesis. Circ Res. 1998 May 4;82(8):871–8.

144. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, et al. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst. 2001 Jul 18;93(14):1054–61.

145. Campbell B, Flatman R, Badrick T, Kanowski D. Problems with High-Sensitivity C-Reactive Protein. Clin Chem. 2003 Jan 1;49(1):201–2.

146. Muthu V, Kozman H, Liu K, Smulyan H, Villarreal D. Cardiac troponins: bench to bedside interpretation in cardiac disease. Am J Med Sci. 2014 Apr;347(4):331–7.

147. De Bacquer D, De Backer G, Kornitzer M, Blackburn H. Prognostic value of ECG findings for total, cardiovascular disease, and coronary heart disease death in men and women. Heart Br Card Soc. 1998 Dec;80(6):570–7.

148. Sun Z, Choo GH, Ng KH. Coronary CT angiography: current status and continuing challenges. Br J Radiol. 2012 May 1;85(1013):495–510.

149. Abramson JD, Rosenberg HG, Jewell N, Wright JM. Should people at low risk of cardiovascular disease take a statin? BMJ. 2013 Oct 22;347:f6123.

150. deGoma EM, Knowles JW, Angeli F, Budoff MJ, Rader DJ. The Evolution and Refinement of Traditional Risk Factors for Cardiovascular Disease. Cardiol Rev. 2012 May;20(3):118–29.

151. Menghini R, Fiorentino L, Casagrande V, Lauro R, Federici M. The role of ADAM17 in metabolic inflammation. Atherosclerosis. 2013 May;228(1):12–7.

152. Scheller J, Chalaris A, Garbers C, Rose-John S. ADAM17: a molecular switch to control inflammation and tissue regeneration. Trends Immunol. 2011 Aug;32(8):380–7.

153. Tashiro H, Shimokawa H, Yamamoto K, Nagano M, Momohara M, Muramatu K, et al. Monocyte-related cytokines in acute myocardial infarction. Am Heart J. 1995 Sep 1;130(3):446–52.

154. Latini R, Bianchi M, Correale E, Dinarello CA, Fantuzzi G, Fresco C, et al. Cytokines in acute myocardial infarction: selective increase in circulating tumor necrosis factor, its soluble receptor, and interleukin-1 receptor antagonist. J Cardiovasc Pharmacol. 1994 Jan;23(1):1–6.

155. Rizza S, Copetti M, Cardellini M, Menghini R, Pecchioli C, Luzi A, et al. A score including ADAM17 substrates correlates to recurring cardiovascular event in subjects with atherosclerosis. Atherosclerosis. 2015 Apr;239(2):459–64.

156. Putko BN, Wang Z, Lo J, Anderson T, Becher H, Dyck JRB, et al. Circulating Levels of Tumor Necrosis Factor-Alpha Receptor 2 Are Increased in Heart Failure with Preserved Ejection Fraction Relative to Heart Failure with Reduced Ejection Fraction: Evidence for a Divergence in Pathophysiology. PLOS ONE. 2014 Jun 12;9(6):e99495.

157. Chemaly M, McGilligan V, Gibson M, Clauss M, Watterson S, Alexander HD, et al. Role of tumour necrosis factor alpha converting enzyme (TACE/ADAM17) and associated proteins in coronary artery disease and cardiac events. Arch Cardiovasc Dis. 2017 Dec;110(12):700–11.

158. Mokou M, Lygirou V, Vlahou A, Mischak H. Proteomics in cardiovascular disease: recent progress and clinical implication and implementation. Expert Rev Proteomics. 2017 Feb 1;14(2):117–36.

159. Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006 Feb;23(2):312–28.

160. Martin-Ramirez J, Hofman M, van den Biggelaar M, Hebbel RP, Voorberg J. Establishment of outgrowth endothelial cells from peripheral blood. Nat Protoc. 2012 Sep;7(9):1709–15.

161. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood. 2004 Nov 1;104(9):2752–60.

162. Frostegård J. Immunity, atherosclerosis and cardiovascular disease. BMC Med. 2013 May 1;11:117.

163. Massberg S, Brand K, Grüner S, Page S, Müller E, Müller I, et al. A Critical Role of Platelet Adhesion in the Initiation of Atherosclerotic Lesion Formation. J Exp Med. 2002 Oct 7;196(7):887–96.

164. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med. 2014 Dec;276(6):618–32.

165. Satoh M, Ishikawa Y, Itoh T, Minami Y, Takahashi Y, Nakamura M. The expression of TNF-alpha converting enzyme at the site of ruptured plaques in patients with acute myocardial infarction. Eur J Clin Invest. 2008 Feb;38(2):97–105.

166. Satoh M, Nakamura M, Satoh H, Saitoh H, Segawa I, Hiramori K. Expression of tumor necrosis factor-alpha--converting enzyme and tumor necrosis factor-alpha in human myocarditis. J Am Coll Cardiol. 2000 Oct;36(4):1288–94.

167. Satoh M, Iwasaka J, Nakamura M, Akatsu T, Shimoda Y, Hiramori K. Increased expression of tumor necrosis factor-alpha converting enzyme and tumor necrosis factor-alpha in peripheral blood mononuclear cells in patients with advanced congestive heart failure. Eur J Heart Fail. 2004 Dec;6(7):869–75.

168. Kaneko H, Anzai T, Horiuchi K, Kohno T, Nagai T, Anzai A, et al. Tumor necrosis factor-α converting enzyme is a key mediator of abdominal aortic aneurysm development. Atherosclerosis. 2011 Oct;218(2):470–8.

169. Takayanagi T, Forrester SJ, Kawai T, Obama T, Tsuji T, Elliott KJ, et al. Vascular ADAM17 as a Novel Therapeutic Target in Mediating Cardiovascular Hypertrophy and Perivascular Fibrosis Induced by Angiotensin IINovelty and Significance. Hypertension. 2016 Oct 1;68(4):949–55.

170. Akatsu T, Nakamura M, Satoh M, Hiramori K. Increased mRNA expression of tumour necrosis factor-alpha and its converting enzyme in circulating leucocytes of patients with acute myocardial infarction. Clin Sci Lond Engl 1979. 2003 Jul;105(1):39–44.

171. Shimoda Y, Satoh M, Nakamura M, Akatsu T, Hiramori K. Activated tumour necrosis factor-alpha shedding process is associated with in-hospital complication in patients with acute myocardial infarction. Clin Sci Lond Engl 1979. 2005 Apr;108(4):339–47.

172. Ling J, Shen L, Liu Q, Xue S, Ma W, Wu H, et al. Changes in platelet GPIbα and ADAM17 during the acute stage of atherosclerotic ischemic stroke among Chinese. J Huazhong Univ Sci Technol Med Sci Hua Zhong Ke Ji Xue Xue Bao Yi Xue Ying Wen Ban Huazhong Keji Daxue Xuebao Yixue Yingdewen Ban. 2013 Jun;33(3):438–42.

173. Groth E, Pruessmeyer J, Babendreyer A, Schumacher J, Pasqualon T, Dreymueller D, et al. Stimulated release and functional activity of surface expressed metalloproteinase ADAM17 in exosomes. Biochim Biophys Acta. 2016 Nov;1863(11):2795–808.

174. Lorenzen I, Lokau J, Korpys Y, Oldefest M, Flynn CM, Künzel U, et al. Control of ADAM17 activity by regulation of its cellular localisation. Sci Rep [Internet]. 2016 Oct 12 [cited 2017 Apr 3];6. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5059621/

175. Canault M, Leroyer AS, Peiretti F, Lesèche G, Tedgui A, Bonardo B, et al. Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. Am J Pathol. 2007 Nov;171(5):1713–23.

176. Bertram A, Lovric S, Engel A, Beese M, Wyss K, Hertel B, et al. Circulating ADAM17 Level Reflects Disease Activity in Proteinase-3 ANCA-Associated Vasculitis. J Am Soc Nephrol JASN. 2015 Nov;26(11):2860–70.

177. Sun Q, Hampel H, Blennow K, Lista S, Levey A, Tang B, et al. Increased plasma TACE activity in subjects with mild cognitive impairment and patients with Alzheimer's disease. J Alzheimers Dis JAD. 2014;41(3):877–86.

178. Petersen JEV, Mkumbaye SI, Vaaben AV, Manjurano A, Lyimo E, Kavishe RA, et al. Plasma Ang2 and ADAM17 levels are elevated during clinical malaria; Ang2 level correlates with severity and expression of EPCR-binding PfEMP1. Sci Rep [Internet]. 2016 Oct 27 [cited 2017 Jan 27];6. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5082358/

179. Jousilahti P, Vartiainen E, Tuomilehto J, Puska P. Sex, Age, Cardiovascular Risk Factors, and Coronary Heart Disease: A Prospective Follow-Up Study of 14 786 Middle-Aged Men and Women in Finland. Circulation. 1999 Mar 9;99(9):1165–72.

180. Kamangar F. Confounding variables in epidemiologic studies: basics and beyond. Arch Iran Med. 2012 Aug;15(8):508–16.

181. Ridker PM, Rifai N, Pfeffer M, Sacks F, Lepage S, Braunwald E. Elevation of Tumor Necrosis Factor-α and Increased Risk of Recurrent Coronary Events After Myocardial Infarction. Circulation. 2000 May 9;101(18):2149–53.

182. Nyström T, Nygren A, Sjöholm A. Increased levels of tumour necrosis factor-alpha (TNF-alpha) in patients with Type II diabetes mellitus after myocardial infarction are related to endothelial dysfunction. Clin Sci Lond Engl 1979. 2006 Jun;110(6):673–81.

183. Wu K, Tian S, Zhou H, Wu Y. Statins protect human endothelial cells from TNF-induced inflammation via ERK5 activation. Biochem Pharmacol. 2013 Jun 15;85(12):1753–60.

184. Cacciapaglia F, Salvatorelli E, Minotti G, Afeltra A, Menna P. Low Level Tumor Necrosis Factor-Alpha Protects Cardiomyocytes Against High Level Tumor Necrosis Factor-Alpha: Brief Insight into a Beneficial Paradox. Cardiovasc Toxicol. 2014 Dec 1;14(4):387–92.

185. Tuomisto K, Jousilahti P, Sundvall J, Pajunen P, Salomaa V. C-reactive protein, interleukin-6 and tumor necrosis factor alpha as predictors of incident coronary and cardiovascular events and total mortality A population-based, prospective study. Thromb Haemost. 2006;95(3):511–8.

186. Habib RH, Dimitrova KR, Badour SA, Yammine MB, El-Hage-Sleiman A-KM, Hoffman DM, et al. CABG Versus PCI. J Am Coll Cardiol. 2015 Sep 29;66(13):1417–27.

187. Hiraoka Y, Yoshida K, Ohno M, Matsuoka T, Kita T, Nishi E. Ectodomain shedding of TNF-alpha is enhanced by nardilysin via activation of ADAM proteases. Biochem Biophys Res Commun. 2008 May 23;370(1):154–8.

188. Hikita A, Tanaka N, Yamane S, Ikeda Y, Furukawa H, Tohma S, et al. Involvement of a disintegrin and metalloproteinase 10 and 17 in shedding of tumor necrosis factor-α. Biochem Cell Biol. 2009 Jun 4;87(4):581–93.

189. Haro H, Crawford HC, Fingleton B, Shinomiya K, Spengler DM, Matrisian LM. Matrix metalloproteinase-7–dependent release of tumor necrosis factor-α in a model of herniated disc resorption. J Clin Invest. 2000 Jan 15;105(2):143–50.

190. Becker-Pauly C, Rose-John S. TNFα cleavage beyond TACE/ADAM17: matrix metalloproteinase 13 is a potential therapeutic target in sepsis and colitis. EMBO Mol Med. 2013 Jul 3;5(7):970–2.

191. Van Zee KJ, Kohno T, Fischer E, Rock CS, Moldawer LL, Lowry SF. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor alpha in vitro and in vivo. Proc Natl Acad Sci U S A. 1992 Jun 1;89(11):4845–9.

192. Tellier E, Canault M, Rebsomen L, Bonardo B, Juhan-Vague I, Nalbone G, et al. The shedding activity of ADAM17 is sequestered in lipid rafts. Exp Cell Res. 2006 Dec 10;312(20):3969–80.

193. Tellier E, Canault M, Poggi M, Bonardo B, Nicolay A, Alessi M-C, et al. HDLs activate ADAM17-dependent shedding. J Cell Physiol. 2008 Mar;214(3):687–93.

194. Fan D, Takawale A, Shen M, Wang W, Wang X, Basu R, et al. Cardiomyocyte A Disintegrin And Metalloproteinase 17 (ADAM17) Is Essential in Post-Myocardial Infarction Repair by Regulating Angiogenesis. Circ Heart Fail. 2015 Sep;8(5):970–9.

195. Lee ACH, Lam JKY, Shiu SWM, Wong Y, Betteridge DJ, Tan KCB. Serum Level of Soluble Receptor for Advanced Glycation End Products Is Associated with A Disintegrin And Metalloproteinase 10 in Type 1 Diabetes. PLOS ONE. 2015 Sep 1;10(9):e0137330.

196. Qi JH, Ebrahem Q, Moore N, Murphy G, Claesson-Welsh L, Bond M, et al. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nat Med. 2003 Apr;9(4):407–15.

197. Canault M, Tellier E, Bonardo B, Mas E, Aumailley M, Juhan-Vague I, et al. FHL2 interacts with both ADAM-17 and the cytoskeleton and regulates ADAM-17 localization and activity. J Cell Physiol. 2006 Aug;208(2):363–72.

198. Peiretti F, Canault M, Deprez-Beauclair P, Berthet V, Bonardo B, Juhan-Vague I, et al. Intracellular maturation and transport of tumor necrosis factor alpha converting enzyme. Exp Cell Res. 2003 May 1;285(2):278–85.

199. Nicolaou A, Zhao Z, Northoff BH, Sass K, Herbst A, Kohlmaier A, et al. Adam17 Deficiency Promotes Atherosclerosis by Enhanced TNFR2 Signaling in MiceHighlights. Arterioscler Thromb Vasc Biol. 2017 Feb 1;37(2):247–57.

200. van der Vorst EPC, Zhao Z, Rami M, Holdt LM, Teupser D, Steffens S, et al. Contrasting effects of myeloid and endothelial ADAM17 on atherosclerosis development. Thromb Haemost. 2017 Feb 28;117(3):644–6.

201. Maretzky T, McIlwain DR, Issuree PDA, Li X, Malapeira J, Amin S, et al. iRhom2 controls the substrate selectivity of stimulated ADAM17-dependent ectodomain shedding. Proc Natl Acad Sci U S A. 2013 Jul 9;110(28):11433–8.

202. Weizman T, Levin I, Zaretsky M, Sagi I, Aharoni A. Increased Potency of a Bi-specific TL1A-ADAM17 (TACE) Inhibitor by Cell Surface Targeting. Front Mol Biosci. 2017;4:61.

203. Berkelmans GFN, van der Graaf Y, Dorresteijn JAN, de Borst GJ, Cramer MJ, Kappelle LJ, et al. Decline in risk of recurrent cardiovascular events in the period 1996 to 2014 partly explained by better treatment of risk factors and less subclinical atherosclerosis. Int J Cardiol. 2018 Jan 15;251:96–102.

204. Piccini JP, Hammill BG, Sinner MF, Hernandez AF, Walkey AJ, Benjamin EJ, et al. Clinical course of atrial fibrillation in older adults: the importance of cardiovascular events beyond stroke. Eur Heart J. 2014 Jan 21;35(4):250–6.

205. Aukrust P, Sandberg WJ, Otterdal K, Vinge LE, Gullestad L, Yndestad A, et al. Tumor necrosis factor superfamily molecules in acute coronary syndromes. Ann Med. 2011 Mar 1;43(2):90–103.

206. Carlsson AC, Östgren CJ, Nystrom FH, Länne T, Jennersjö P, Larsson A, et al. Association of soluble tumor necrosis factor receptors 1 and 2 with nephropathy, cardiovascular events, and total mortality in type 2 diabetes. Cardiovasc Diabetol. 2016 Feb 29;15:40.

207. Safranow K, Dziedziejko V, Rzeuski R, Czyzycka E, Wojtarowicz A, Bińczak-Kuleta A, et al. Plasma concentrations of TNF-alpha and its soluble receptors sTNFR1 and sTNFR2 in patients with coronary artery disease. Tissue Antigens. 2009 Nov;74(5):386–92.

208. Cabal-Hierro L, Lazo PS. Signal transduction by tumor necrosis factor receptors. Cell Signal. 2012 Jun;24(6):1297–305.

209. Luo D, Luo Y, He Y, Zhang H, Zhang R, Li X, et al. Differential Functions of Tumor Necrosis Factor Receptor 1 and 2 Signaling in Ischemia-Mediated Arteriogenesis and Angiogenesis. Am J Pathol. 2006 Nov;169(5):1886–98.

210. Luo Y, Xu Z, Wan T, He Y, Jones D, Zhang H, et al. Endothelial-specific transgenesis of TNFR2 promotes adaptive arteriogenesis and angiogenesis. Arterioscler Thromb Vasc Biol. 2010 Jul;30(7):1307–14.

211. Chen G, Goeddel DV. TNF-R1 Signaling: A Beautiful Pathway. Science. 2002 May 31;296(5573):1634–5.

212. Tartaglia LA, Pennica D, Goeddel DV. Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J Biol Chem. 1993 Sep 5;268(25):18542–8.

213. Rocha NP, Teixeira AL, Scalzo PL, Barbosa IG, de Sousa MS, Morato IB, et al. Plasma levels of soluble tumor necrosis factor receptors are associated with cognitive performance in Parkinson's disease. Mov Disord Off J Mov Disord Soc. 2014 Apr;29(4):527–31.

214. Gohda T, Maruyama S, Kamei N, Yamaguchi S, Shibata T, Murakoshi M, et al. Circulating TNF Receptors 1 and 2 Predict Mortality in Patients with End-stage Renal Disease Undergoing Dialysis. Sci Rep. 2017 Mar 3;7:srep43520.

215. Oregón-Romero E, Mercado MV-D, Navarro-Hernández RE, Torres-Carrillo N, Martínez-Bonilla G, Estrada-García I, et al. Tumor necrosis factor receptor 2 M196R polymorphism in rheumatoid arthritis and osteoarthritis: relationship with sTNFR2 levels and clinical features. Rheumatol Int. 2006 Nov 1;27(1):53–9.

216. Straczkowski M, Kowalska I, Stepien A, Dzienis-Straczkowska S, Szelachowska M, Kinalska I. Increased plasma-soluble tumor necrosis factor-alpha receptor 2 level in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes Care. 2002 Oct;25(10):1824–8.

217. Black RA. TIMP3 checks inflammation. Nat Genet. 2004 Sep;36(9):934–5.

218. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett. 1998 Sep 11;435(1):39–44.

219. Chavey C, Mari B, Monthouel M-N, Bonnafous S, Anglard P, Van Obberghen E, et al. Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. J Biol Chem. 2003 Apr 4;278(14):11888–96.

220. Menghini R, Casagrande V, Menini S, Marino A, Marzano V, Hribal ML, et al. TIMP3 overexpression in macrophages protects from insulin resistance, adipose inflammation, and nonalcoholic fatty liver disease in mice. Diabetes. 2012 Feb;61(2):454–62.

221. Cardellini M, Menghini R, Luzi A, Davato F, Cardolini I, D'Alfonso R, et al. Decreased IRS2 and TIMP3 expression in monocytes from offspring of type 2 diabetic patients is correlated with insulin resistance and increased intima-media thickness. Diabetes. 2011 Dec;60(12):3265–70.

222. Valgimigli M, Ceconi C, Malagutti P, Merli E, Soukhomovskaia O, Francolini G, et al. Tumor necrosis factor-alpha receptor 1 is a major predictor of mortality and new-onset heart failure in patients with acute myocardial infarction: the Cytokine-Activation and Long-Term Prognosis in Myocardial Infarction (C-ALPHA) study. Circulation. 2005 Feb 22;111(7):863–70.

223. Ping Z, Aiqun M, Jiwu L, Liang S. TNF Receptor 1/2 Predict Heart Failure Risk in Type 2 Diabetes Mellitus Patients. Int Heart J. 2017 Apr 6;58(2):245–9.

224. Bae E, Cha R-H, Kim YC, An JN, Kim DK, Yoo KD, et al. Circulating TNF receptors predict cardiovascular disease in patients with chronic kidney disease. Medicine (Baltimore)

[Internet]. 2017 May 12 [cited 2017 Jun 29];96(19). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5428576/

225. Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U. Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. J Neurosci Off J Soc Neurosci. 2002 Apr 1;22(7):RC216.

226. Sama DM, Abdul HM, Furman JL, Artiushin IA, Szymkowski DE, Scheff SW, et al. Inhibition of Soluble Tumor Necrosis Factor Ameliorates Synaptic Alterations and Ca2+ Dysregulation in Aged Rats. PLOS ONE. 2012 May 29;7(5):e38170.

227. Higuchi Y, McTiernan CF, Frye CB, McGowan BS, Chan TO, Feldman AM. Tumor necrosis factor receptors 1 and 2 differentially regulate survival, cardiac dysfunction, and remodeling in transgenic mice with tumor necrosis factor-alpha-induced cardiomyopathy. Circulation. 2004 Apr 20;109(15):1892–7.

228. MacEwan DJ. TNF receptor subtype signalling: Differences and cellular consequences. Cell Signal. 2002 Jun 1;14(6):477–92.

229. Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: structure and function. Trends Cell Biol. 1995 Oct 1;5(10):392–9.

230. McCoy MK, Tansey MG. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J Neuroinflammation. 2008 Oct 17;5:45.

231. Basu R, Lee J, Morton JS, Takawale A, Fan D, Kandalam V, et al. TIMP3 is the primary TIMP to regulate agonist-induced vascular remodelling and hypertension. Cardiovasc Res. 2013 Jun 1;98(3):360–71.

232. Ma R, Gu B, Gu Y, Groome LJ, Wang Y. Down-regulation of TIMP3 leads to increase in TACE expression and TNFα production by placental trophoblast cells. Am J Reprod Immunol N Y N 1989. 2014 May;71(5):427–33.

233. Stöhr R, Cavalera M, Menini S, Mavilio M, Casagrande V, Rossi C, et al. Loss of TIMP3 exacerbates atherosclerosis in ApoE null mice. Atherosclerosis. 2014 Aug;235(2):438–43.

234. Martz L. Taking TIMP3 to heart. SciBX Sci-Bus Exch [Internet]. 2014 Mar 6 [cited 2017 Jun 29];7(9). Available from: http://www.nature.com/scibx/journal/v7/n9/full/scibx.2014.246.html

235. Xu P, Liu J, Sakaki-Yumoto M, Derynck R. TACE activation by MAPK-mediated regulation of cell surface dimerization and TIMP3 association. Sci Signal. 2012 May 1;5(222):ra34.

236. Zheng Y, Schlondorff J, Blobel CP. Evidence for regulation of the tumor necrosis factor alpha-convertase (TACE) by protein-tyrosine phosphatase PTPH1. J Biol Chem. 2002 Nov 8;277(45):42463–70.

237. Bernot D, Barruet E, Poggi M, Bonardo B, Alessi M-C, Peiretti F. Down-regulation of Tissue Inhibitor of Metalloproteinase-3 (TIMP-3) Expression Is Necessary for Adipocyte Differentiation. J Biol Chem. 2010 Feb 26;285(9):6508–14.

238. Stöhr R, Kappel BA, Carnevale D, Cavalera M, Mavilio M, Arisi I, et al. TIMP3 interplays with apelin to regulate cardiovascular metabolism in hypercholesterolemic mice. Mol Metab. 2015 Oct;4(10):741–52.

239. Ridker PM. Closing the loop on inflammation and atherothrombosis: why perform the CIRT and CANTOS trials? Trans Am Clin Climatol Assoc. 2013;124:174–90.

240. Hartman J, Frishman WH. Inflammation and atherosclerosis: a review of the role of interleukin-6 in the development of atherosclerosis and the potential for targeted drug therapy. Cardiol Rev. 2014 Jun;22(3):147–51.

241. Simon AD, Yazdani S, Wang W, Schwartz A, Rabbani LE. Elevated plasma levels of interleukin-2 and soluble IL-2 receptor in ischemic heart disease. Clin Cardiol. 2001 Mar;24(3):253–6.

242. Kanda T, Takahashi T. Interleukin-6 and cardiovascular diseases. Jpn Heart J. 2004 Mar;45(2):183–93.

243. Zeng Z, Yu K, Chen L, Li W, Xiao H, Huang Z. Interleukin-2/Anti-Interleukin-2 Immune Complex Attenuates Cardiac Remodeling after Myocardial Infarction through Expansion of Regulatory T Cells [Internet]. Journal of Immunology Research. 2016 [cited 2017 Oct 7]. Available from: https://www.hindawi.com/journals/jir/2016/8493767/

244. Wang H, Hou L, Kwak D, Fassett J, Xu X, Chen A, et al. Increasing Regulatory T Cells With Interleukin-2 and Interleukin-2 Antibody Complexes Attenuates Lung Inflammation and Heart Failure Progression. Hypertension. 2016 Jan 1;HYPERTENSIONAHA.116.07084.

245. Apostolakis S, Vogiatzi K, Amanatidou V, Spandidos DA. Interleukin 8 and cardiovascular disease. Cardiovasc Res. 2009 Dec 1;84(3):353–60.

246. Romuk E, Skrzep-Poloczek B, Wojciechowska C, Tomasik A, Birkner E, Wodniecki J, et al. Selectin-P and interleukin-8 plasma levels in coronary heart disease patients. Eur J Clin Invest. 2002 Sep 1;32(9):657–61.

247. Matic M, Simon SR. Effects of gamma interferon on release of tumor necrosis factor alpha from lipopolysaccharide-tolerant human monocyte-derived macrophages. Infect Immun. 1992 Sep;60(9):3756–62.

248. Voloshyna I, Littlefield MJ, Reiss AB. Atherosclerosis and interferon-γ: New insights and therapeutic targets. Trends Cardiovasc Med [Internet]. 2014 Jan [cited 2017 Aug 28];24(1). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3844070/

249. Girndt M, Köhler H. Interleukin-10 (IL-10): an update on its relevance for cardiovascular risk. Nephrol Dial Transplant. 2003 Oct 1;18(10):1976–9.

250. Hofmann U, Knorr S, Vogel B, Weirather J, Frey A, Ertl G, et al. Interleukin-13 Deficiency Aggravates Healing and Remodeling in Male Mice After Experimental Myocardial InfarctionCLINICAL PERSPECTIVE. Circ Heart Fail. 2014 Sep 1;7(5):822–30.

251. Peng H, Sarwar Z, Yang X-P, Peterson EL, Xu J, Janic B, et al. Profibrotic Role for Interleukin-4 in Cardiac Remodeling and Dysfunction. Hypertens Dallas Tex 1979. 2015 Sep;66(3):582–9.

252. Mali V, Tennant W, Haddox S, Matrougui K, Belmadani S. Interleukin-12 Disruption Protects Myocardium from Ischemia-Reperfusion Injury in Type 2 Diabetic Mice. FASEB J. 2017 Apr 1;31(1 Supplement):680.4-680.4.

253. Lee TS, Yen HC, Pan CC, Chau LY. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. Arterioscler Thromb Vasc Biol. 1999 Mar;19(3):734–42.

254. Moss JW, Ramji DP. Interferon-γ: Promising therapeutic target in atherosclerosis. World J Exp Med. 2015 Aug 20;5(3):154–9.

255. Miyao Y, Yasue H, Ogawa H, Misumi I, Masuda T, Sakamoto T, et al. Elevated plasma interleukin-6 levels in patients with acute myocardial infarction. Am Heart J. 1993 Dec 1;126(6):1299–304.

256. Danesh J, Kaptoge S, Mann AG, Sarwar N, Wood A, Angleman SB, et al. Long-Term Interleukin-6 Levels and Subsequent Risk of Coronary Heart Disease: Two New Prospective Studies and a Systematic Review. PLoS Med [Internet]. 2008 Apr [cited 2017 Aug 30];5(4). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2288623/

257. Hoejberg L, Bastholt L, Johansen JS, Christensen IJ, Gehl J, Schmidt H. Serum interleukin-6 as a prognostic biomarker in patients with metastatic melanoma. Melanoma Res. 2012 Aug;22(4):287–93.

258. Paquette SG, Banner D, Zhao Z, Fang Y, Huang SSH, León AJ, et al. Interleukin-6 Is a Potential Biomarker for Severe Pandemic H1N1 Influenza A Infection. PLOS ONE. 2012 Jun 5;7(6):e38214.

259. Takeuchi T, Miyasaka N, Tatsuki Y, Yano T, Yoshinari T, Abe T, et al. Baseline tumour necrosis factor alpha levels predict the necessity for dose escalation of infliximab therapy in patients with rheumatoid arthritis. Ann Rheum Dis. 2011 Jul;70(7):1208–15.

260. Hober D, Nguyen TL, Shen L, Ha DQ, Huong VT, Benyoucef S, et al. Tumor necrosis factor alpha levels in plasma and whole-blood culture in dengue-infected patients: relationship between virus detection and pre-existing specific antibodies. J Med Virol. 1998 Mar;54(3):210–8.

261. Ding X, Yang S, Li W, Liu Y, Li Z, Zhang Y, et al. The Potential Biomarker Panels for Identification of Major Depressive Disorder (MDD) Patients with and without Early Life Stress (ELS) by Metabonomic Analysis. PLOS ONE. 2014 May 28;9(5):e97479.

262. Fung KYC, Tabor B, Buckley MJ, Priebe IK, Purins L, Pompeia C, et al. Blood-based protein biomarker panel for the detection of colorectal cancer. PloS One. 2015;10(3):e0120425.

263. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017 Aug 27;0(0):null.

264. Bujak M, Frangogiannis NG. The role of IL-1 in the pathogenesis of heart disease. Arch Immunol Ther Exp (Warsz). 2009 Jun;57(3):165–76.

265. Di Iorio A, Ferrucci L, Sparvieri E, Cherubini A, Volpato S, Corsi A, et al. Serum IL-1beta levels in health and disease: a population-based study. “The InCHIANTI study.” Cytokine. 2003 Jun 21;22(6):198–205.

266. Osuch M, Szygula-Jurkiewicz B, Szkodzinski J, Wojnicz R, Lekston A, Polonski L, et al. The role of IL-4 and IFN-gamma as prognostic factors of major adverse cardiac events in patients with ST-elevation myocardial infarction assigned primary percutaneous coronary intervention. 2006 Dec 4 [cited 2017 Aug 30]; Available from: http://www.endocrine-abstracts.org/ea/0011/ea0011p218.htm

267. Ikeda U, Ito T, Shimada K. Interleukin-6 and acute coronary syndrome. Clin Cardiol. 2001 Nov;24(11):701–4.

268. Pedersen BK, Steensberg A, Schjerling P. Muscle-derived interleukin-6: possible biological effects. J Physiol. 2001 Oct 15;536(Pt 2):329–37.

269. Karpiński L, Płaksej R, Kosmala W, Witkowska M. Serum levels of interleukin-6, interleukin-10 and C-reactive protein in relation to left ventricular function in patients with myocardial infarction treated with primary angioplasty. Kardiol Pol. 2008 Dec;66(12):1279–85.

270. Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J Physiol. 1999 Feb 15;515 (Pt 1):287–91.

271. López-Cuenca Á, Manzano-Fernández S, Lip GYH, Casas T, Sánchez-Martínez M, Mateo-Martínez A, et al. Interleukin-6 and High-sensitivity C-reactive Protein for the Prediction of Outcomes in Non-ST-segment Elevation Acute Coronary Syndromes. Rev Esp Cardiol. 2013 Jan 3;66(03):185–92.

272. Zykov MV, Barbarash OL, Kashtalap VV, Kutikhin AG, Barbarash LS. Interleukin-12 serum level has prognostic value in patients with ST-segment elevation myocardial infarction. Heart Lung J Crit Care. 2016 Aug;45(4):336–40.

273. Mishra M, Kumar H, Bajpai S, Singh RK, Tripathi K. Level of serum IL-12 and its correlation with endothelial dysfunction, insulin resistance, proinflammatory cytokines and lipid profile in newly diagnosed type 2 diabetes. Diabetes Res Clin Pract. 2011 Nov;94(2):255–61.

274. Tzellos T, Kyrgidis A, Zouboulis CC. Re-evaluation of the risk for major adverse cardiovascular events in patients treated with anti-IL-12/23 biological agents for chronic plaque psoriasis: a meta-analysis of randomized controlled trials. J Eur Acad Dermatol Venereol JEADV. 2013 May;27(5):622–7.

275. Robbins JM, Webb DA, Sciamanna CN. Cardiovascular comorbidities among public health clinic patients with diabetes: the Urban Diabetics Study. BMC Public Health. 2005 Feb 8;5:15.

276. Collins AJ, Li S, Gilbertson DT, Liu J, Chen S-C, Herzog CA. Chronic kidney disease and cardiovascular disease in the Medicare population: Management of comorbidities in kidney disease in the 21st century: Anemia and bone disease. Kidney Int. 2003 Nov 1;64(Supplement 87):S24–31.

277. Malik S, Wong ND, Franklin SS, Kamath TV, L’Italien GJ, Pio JR, et al. Impact of the Metabolic Syndrome on Mortality From Coronary Heart Disease, Cardiovascular

Disease, and All Causes in United States Adults. Circulation. 2004 Sep 7;110(10):1245–50.

278. Elderon L, Whooley MA. Depression and Cardiovascular Disease. Prog Cardiovasc Dis. 2013 May 1;55(6):511–23.

279. Hess JW, Macdonald RP, Frederick RJ, Jones RN, Neely J, Gross D. SERUM CREATINE PHOSPHOKINASE (CPK) ACTIVITY IN DISORDERS OF HEART AND SKELETAL MUSCLE. Ann Intern Med. 1964 Dec;61:1015–28.

280. Antman EM, Tanasijevic MJ, Thompson B, Schactman M, McCabe CH, Cannon CP, et al. Cardiac-Specific Troponin I Levels to Predict the Risk of Mortality in Patients with Acute Coronary Syndromes. N Engl J Med. 1996 Oct 31;335(18):1342–9.

281. Stubbs P, Collinson P, Moseley D, Greenwood T, Noble M. Prospective study of the role of cardiac troponin T in patients admitted with unstable angina. BMJ. 1996 Aug 3;313(7052):262–4.

282. de Lemos JA, Morrow DA, Bentley JH, Omland T, Sabatine MS, McCabe CH, et al. The prognostic value of B-type natriuretic peptide in patients with acute coronary syndromes. N Engl J Med. 2001 Oct 4;345(14):1014–21.

283. Gibson DS, Drain S, Kelly C, McGilligan V, McClean P, Atkinson SD, et al. Coincidence versus consequence: opportunities in multi-morbidity research and inflammation as a pervasive feature. Expert Rev Precis Med Drug Dev. 2017 May 4;2(3):147–56.

284. Dawber TR, Meadors GF, Moore FE. Epidemiological Approaches to Heart Disease: The Framingham Study. Am J Public Health Nations Health. 1951 Mar;41(3):279–86.

285. Karmali KN, Persell SD, Perel P, Lloyd-Jones DM, Berendsen MA, Huffman MD. Risk scoring for the primary prevention of cardiovascular disease. Cochrane Database Syst Rev. 2017 14;3:CD006887.

286. Greenland P, Alpert JS, Beller GA, Benjamin EJ, Budoff MJ, Fayad ZA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol. 2010 Dec 14;56(25):e50-103.

287. Khot UN, Khot MB, Bajzer CT, Sapp SK, Ohman EM, Brener SJ, et al. Prevalence of conventional risk factors in patients with coronary heart disease. JAMA. 2003 Aug 20;290(7):898–904.

288. Cooper JA, Miller GJ, Humphries SE. A comparison of the PROCAM and Framingham point-scoring systems for estimation of individual risk of coronary heart disease in the Second Northwick Park Heart Study. Atherosclerosis. 2005 Jul;181(1):93–100.

289. MacLellan WR, Wang Y, Lusis AJ. Systems-based approaches to cardiovascular disease. Nat Rev Cardiol [Internet]. 2012 Jan 10 [cited 2017 Sep 26];9(3). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3823242/

290. Hingorani AD, Sofat R, Morris RW, Whincup P, Lowe GD, Mindell J, et al. Is it important to measure or reduce C-reactive protein in people at risk of cardiovascular disease? Eur Heart J. 2012 Sep;33(18):2258–64.

291. Póvoa P, Coelho L, Almeida E, Fernandes A, Mealha R, Moreira P, et al. C-reactive protein as a marker of infection in critically ill patients. Clin Microbiol Infect. 2005 Feb 1;11(2):101–8.

292. Kip KE, Marroquin OC, Shaw LJ, Arant CB, Wessel TR, Olson MB, et al. Global inflammation predicts cardiovascular risk in women: a report from the Women's Ischemia Syndrome Evaluation (WISE) study. Am Heart J. 2005 Nov;150(5):900–6.

293. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Münzel T, et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation. 2003 Sep 23;108(12):1440–5.

294. Bodí V, Sanchis J, Llàcer A, Fácila L, Núñez J, Pellicer M, et al. Multimarker risk strategy for predicting 1-month and 1-year major events in non-ST-elevation acute coronary syndromes. Am Heart J. 2005 Feb;149(2):268–74.

295. Heeschen C, Dimmeler S, Hamm CW, Fichtlscherer S, Simoons ML, Zeiher AM, et al. Pregnancy-associated plasma protein-A levels in patients with acute coronary syndromes: comparison with markers of systemic inflammation, platelet activation, and myocardial necrosis. J Am Coll Cardiol. 2005 Jan 18;45(2):229–37.

296. James SK, Lindahl B, Siegbahn A, Stridsberg M, Venge P, Armstrong P, et al. N-terminal pro-brain natriuretic peptide and other risk markers for the separate prediction of mortality and subsequent myocardial infarction in patients with unstable coronary artery disease: a Global Utilization of Strategies To Open occluded arteries (GUSTO)-IV substudy. Circulation. 2003 Jul 22;108(3):275–81.

297. Abbas A, Aukrust P, Russell D, Krohg-Sørensen K, Almås T, Bundgaard D, et al. Matrix Metalloproteinase 7 Is Associated with Symptomatic Lesions and Adverse Events in Patients with Carotid Atherosclerosis. PLOS ONE. 2014 Jan 6;9(1):e84935.

298. Pirillo A, Catapano AL. Soluble Lectin-Like Oxidized Low Density Lipoprotein Receptor-1 as a Biochemical Marker for Atherosclerosis-Related Diseases. Dis Markers. 2013;35(5):413–8.

299. Haywood J, Qi J, Chen C-C, Lu G, Liu Y, Yan J, et al. Structural basis of collagen recognition by human osteoclast-associated receptor and design of osteoclastogenesis inhibitors. Proc Natl Acad Sci. 2016 Jan 26;113(4):1038–43.

300. Reithmeier A, Panizza E, Krumpel M, Orre LM, Branca RMM, Lehtiö J, et al. Tartrate-resistant acid phosphatase (TRAP/ACP5) promotes metastasis-related properties via TGFβ2/TβR and CD44 in MDA-MB-231 breast cancer cells. BMC Cancer. 2017 Sep 15;17:650.

301. Engelen L, Ferreira I, Brouwers O, Henry RMA, Dekker JM, Nijpels G, et al. Polymorphisms in glyoxalase 1 gene are not associated with vascular complications: the Hoorn and CoDAM studies. J Hypertens. 2009 Jul;27(7):1399–403.

302. Amor M, Moreno-Viedma V, Sarabi A, Grün NG, Itariu B, Leitner L, et al. Identification of Matrix Metalloproteinase-12 as a Candidate Molecule for Prevention and Treatment of Cardiometabolic Disease. Mol Med. 2016 Jun 30;22:487–96.

303. Nénan S, Boichot E, Lagente V, Bertrand CP. Macrophage elastase (MMP-12): a pro-inflammatory mediator? Mem Inst Oswaldo Cruz. 2005 Mar;100 Suppl 1:167–72.

304. Singer BB, Opp L, Heinrich A, Schreiber F, Binding-Liermann R, Berrocal-Almanza LC, et al. Soluble CEACAM8 Interacts with CEACAM1 Inhibiting TLR2-Triggered Immune Responses. PLOS ONE. 2014 Apr 17;9(4):e94106.

305. Yamac AH, Sevgili E, Kucukbuzcu S, Nasifov M, Ismailoglu Z, Kilic E, et al. Role of cathepsin D activation in major adverse cardiovascular events and new-onset heart failure after STEMI. Herz. 2015 Sep;40(6):912–20.

306. Page G, Miossec P. RANK and RANKL expression as markers of dendritic cell-T cell interactions in paired samples of rheumatoid synovium and lymph nodes. Arthritis Rheum. 2005 Aug;52(8):2307–12.

307. Bhandari SK, Batech M, Shi J, Jacobsen SJ, Sim JJ. Plasma renin activity and risk of cardiovascular and mortality outcomes among individuals with elevated and nonelevated blood pressure. Kidney Res Clin Pract. 2016 Dec;35(4):219–28.

308. Kempf T, Wollert KC. Growth differentiation factor-15: a new biomarker in cardiovascular disease. Herz. 2009 Dec;34(8):594–9.

309. Kowalska K, Socha E, Milnerowicz H. Review: The role of paraoxonase in cardiovascular diseases. Ann Clin Lab Sci. 2015;45(2):226–33.

310. Greenwood C, Ruff D, Kirvell S, Johnson G, Dhillon HS, Bustin SA. Proximity assays for sensitive quantification of proteins. Biomol Detect Quantif. 2015 Jun 1;4(Supplement C):10–6.

311. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. PanelomiX: A threshold-based algorithm to create panels of biomarkers. Transl Proteomics. 2013 Jan 1;1(1):57–64.

312. Akyol O, Akyol S, Chen C-H. Update on ADAMTS13 and VWF in cardiovascular and hematological disorders. Clin Chim Acta. 2016 Dec 1;463:109–18.

313. Geoffrion M, Du X, Irshad Z, Vanderhyden BC, Courville K, Sui G, et al. Differential effects of glyoxalase 1 overexpression on diabetic atherosclerosis and renal dysfunction in streptozotocin-treated, apolipoprotein E-deficient mice. Physiol Rep [Internet]. 2014 Jun 11 [cited 2017 Sep 26];2(6). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4208644/

314. Schmitt MMN, Megens RTA, Zernecke A, Bidzhekov K, van den Akker NM, Rademakers T, et al. Endothelial junctional adhesion molecule-a guides monocytes into flow-dependent predilection sites of atherosclerosis. Circulation. 2014 Jan 7;129(1):66–76.

315. Zernecke A, Shagdarsuren E, Weber C. Chemokines in Atherosclerosis: An Update. Arterioscler Thromb Vasc Biol. 2008 Nov 1;28(11):1897–908.

316. Hinderer C, Bell P, Gurda BL, Wang Q, Louboutin J-P, Zhu Y, et al. Liver-directed gene therapy corrects cardiovascular lesions in feline mucopolysaccharidosis type I. Proc Natl Acad Sci U S A. 2014 Oct 14;111(41):14894–9.

317. Røysland R, Bonaca MP, Omland T, Sabatine M, Murphy SA, Scirica BM, et al. Osteoprotegerin and cardiovascular mortality in patients with non-ST elevation acute coronary syndromes. Heart. 2012 May 15;98(10):786–91.

318. Winckers K, ten Cate H, Hackeng TM. The role of tissue factor pathway inhibitor in atherosclerosis and arterial thrombosis. Blood Rev. 2013 May;27(3):119–32.

319. An G, Wang H, Tang R, Yago T, McDaniel JM, McGee S, et al. P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice. Circulation. 2008 Jun 24;117(25):3227–37.

320. de Luis DA, Sagrado MG, Aller R, Izaola O, Conde R, Romero E. Ala54Thr polymorphism of fatty acid binding protein 2, role on insulin resistance and cardiovascular risk factors in presurgical morbid obesity patients. Obes Surg. 2009 Dec;19(12):1691–6.

321. Rurali E, Noris M, Chianca A, Donadelli R, Banterla F, Galbusera M, et al. ADAMTS13 predicts renal and cardiovascular events in type 2 diabetic patients and response to therapy. Diabetes. 2013 Oct;62(10):3599–609.

322. Waller AH, Sanchez-Ross M, Kaluski E, Klapholz M. Osteopontin in cardiovascular disease: a potential therapeutic target. Cardiol Rev. 2010 Jun;18(3):125–31.

323. Májek P, Reicheltová Z, Suttnar J, Malý M, Oravec M, Pečánková K, et al. Plasma proteome changes in cardiovascular disease patients: novel isoforms of apolipoprotein A1. J Transl Med. 2011 Jun 1;9:84.

324. Gu H, Xie M, Xu L, Zheng X, Yang Y, Lv X. The protective role of interleukin-18 binding protein in a murine model of cardiac ischemia/reperfusion injury. Transpl Int Off J Eur Soc Organ Transplant. 2015 Dec;28(12):1436–44.

325. Makki N, Thiel KW, Miller FJ. The Epidermal Growth Factor Receptor and Its Ligands in Cardiovascular Disease. Int J Mol Sci. 2013 Oct 15;14(10):20597–613.

326. Zhu H, Guo D, Li K, Yan W, Tan Y, Wang X, et al. PROSTASIN: A POSSIBLE CANDIDATE GENE FOR HUMAN HYPERTENSION. Am J Hypertens [Internet]. 2008 Sep [cited 2017 Sep 22];21(9). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3812941/

327. Tamaki S, Mano T, Sakata Y, Ohtani T, Takeda Y, Kamimura D, et al. Interleukin-16 promotes cardiac fibrosis and myocardial stiffening in heart failure with preserved ejection fraction. PloS One. 2013;8(7):e68893.

328. Franceschini N, Muallem H, Rose KM, Boerwinkle E, Maeda N. LDL Receptor Polymorphisms and the Risk of Coronary Heart Disease: the Atherosclerosis Risk in Communities Study. J Thromb Haemost JTH. 2009 Mar;7(3):496–8.

329. Goldstein JL, Brown MS. The LDL Receptor. Arterioscler Thromb Vasc Biol. 2009 Apr 1;29(4):431–8.

330. Nowak N, Skupien J, Niewczas MA, Yamanouchi M, Major M, Croall S, et al. Increased plasma kidney injury molecule-1 suggests early progressive renal decline in non-proteinuric patients with type 1 diabetes. Kidney Int. 2016 Feb;89(2):459–67.

331. Carlsson AC, Larsson A, Helmersson-Karlqvist J, Lind L, Ingelsson E, Larsson TE, et al. Urinary Kidney Injury Molecule-1 and the Risk of Cardiovascular Mortality in Elderly Men. Clin J Am Soc Nephrol CJASN. 2014 Aug 7;9(8):1393–401.

332. Metcalfe KA, Hitman GA, Pociot F, Bergholdt R, Tuomilehto-Wolf E, Tuomilehto J, et al. An association between type 1 diabetes and the interleukin-1 receptor type 1 gene. The DiMe Study Group. Childhood Diabetes in Finland. Hum Immunol. 1996 Nov;51(1):41–8.

333. Bujak M, Dobaczewski M, Chatila K, Mendoza LH, Li N, Reddy A, et al. Interleukin-1 Receptor Type I Signaling Critically Regulates Infarct Healing and Cardiac Remodeling. Am J Pathol. 2008 Jul;173(1):57–67.

334. Velde AR van der, Meijers WC, Ho JE, Brouwers FP, Rienstra M, Bakker SJL, et al. Serial galectin-3 and future cardiovascular disease in the general population. Heart. 2016 Apr 15;heartjnl-2015-308975.

335. Li S, Bi P, Zhao W, Lian Y, Zhu H, Xu D, et al. Prognostic Utility of Fatty Acid-Binding Protein 4 in Patients with Type 2 Diabetes and Acute Ischemic Stroke. Neurotox Res. 2017 Aug 11;

336. Pugliese G, Iacobini C, Ricci C, Blasetti Fantauzzi C, Menini S. Galectin-3 in diabetic patients. Clin Chem Lab Med. 2014 Oct;52(10):1413–23.

337. Furuhashi M, Saitoh S, Shimamoto K, Miura T. Fatty Acid-Binding Protein 4 (FABP4): Pathophysiological Insights and Potent Clinical Biomarker of Metabolic and Cardiovascular Diseases. Clin Med Insights Cardiol. 2014;8(Suppl 3):23–33.

338. Christlieb AR, Kaldany A, D'Elia JA. Plasma renin activity and hypertension in diabetes mellitus. Diabetes. 1976 Oct;25(10):969–74.

339. Marsillach J, Becker JO, Vaisar T, Hahn BH, Brunzell JD, Furlong CE, et al. Paraoxonase-3 is depleted from the high-density lipoproteins of autoimmune disease patients with subclinical atherosclerosis. J Proteome Res. 2015 May 1;14(5):2046–54.

340. Wu M-L, Ho Y-C, Lin C-Y, Yet S-F. Heme oxygenase-1 in inflammation and cardiovascular disease. Am J Cardiovasc Dis. 2011 Jul 28;1(2):150–8.

341. Tiwari S, Ndisang JF. The heme oxygenase system and type-1 diabetes. Curr Pharm Des. 2014;20(9):1328–37.

342. Wang J-S, Sheu WH-H, Lee W-J, Lee I-T, Lin S-Y, Lee W-L, et al. Associations of fibroblast growth factor 21 with cardiovascular risk and β-cell function in patients who had no history of diabetes. Clin Chim Acta Int J Clin Chem. 2017 Sep;472:80–5.

343. Chou R-H, Huang P-H, Hsu C-Y, Chang C-C, Leu H-B, Huang C-C, et al. Circulating Fibroblast Growth Factor 21 is Associated with Diastolic Dysfunction in Heart Failure Patients with Preserved Ejection Fraction. Sci Rep. 2016 Sep 21;6:srep33953.

344. Wong HK, Cheung TT, Cheung BMY. Adrenomedullin and cardiovascular diseases. JRSM Cardiovasc Dis [Internet]. 2012 Aug 10 [cited 2017 Sep 15];1(5). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3738363/

345. Gómez AM, Guatimosim S, Dilly KW, Vassort G, Lederer WJ. Heart Failure After Myocardial Infarction: Altered Excitation-Contraction Coupling. Circulation. 2001 Aug 7;104(6):688–93.

346. Zoeller JJ, McQuillan A, Whitelock J, Ho S-Y, Iozzo RV. A central function for perlecan in skeletal muscle and cardiovascular development. J Cell Biol. 2008 Apr 21;181(2):381–94.

347. Segev A, Nili N, Strauss BH. The role of perlecan in arterial injury and angiogenesis. Cardiovasc Res. 2004 Sep 1;63(4):603–10.

348. Bobik A, Kalinina N. Tumor necrosis factor receptor and ligand superfamily family members TNFRSF14 and LIGHT: new players in human atherogenesis. Arterioscler Thromb Vasc Biol. 2001 Dec;21(12):1873–5.

349. Meijers B, Poesen R, Claes K, Dietrich R, Bammens B, Sprangers B, et al. Soluble urokinase receptor is a biomarker of cardiovascular disease in chronic kidney disease. Kidney Int. 2015 Jan;87(1):210–6.

350. Grandoch M, Feldmann K, Göthert JR, Dick LS, Homann S, Klatt C, et al. Deficiency in lymphotoxin β receptor protects from atherosclerosis in apoE-deficient mice. Circ Res. 2015 Apr 10;116(8):e57-68.

351. Hohensinner PJ, Niessner A, Huber K, Weyand CM, Wojta J. Inflammation and Cardiac Outcome. Curr Opin Infect Dis. 2011 Jun;24(3):259–64.

352. Poldervaart JM, Langedijk M, Backus BE, Dekker IMC, Six AJ, Doevendans PA, et al. Comparison of the GRACE, HEART and TIMI score to predict major adverse cardiac events in chest pain patients at the emergency department. Int J Cardiol. 2017 Jan 15;227(Supplement C):656–61.

353. Neirynck N, Glorieux G, Schepers E, Verbeke F, Vanholder R. Soluble Tumor Necrosis Factor Receptor 1 and 2 Predict Outcomes in Advanced Chronic Kidney Disease: A Prospective Cohort Study. PLoS ONE [Internet]. 2015 Mar 30 [cited 2017 Sep 7];10(3). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4379033/

354. Zhao L, Wang X, Yang Y. Association between interleukin-6 and the risk of cardiac events measured by coronary computed tomography angiography. Int J Cardiovasc Imaging. 2017 Aug;33(8):1237–44.

355. Chen S-L, Liu Y, Lin L, Ye F, Zhang J-J, Tian N-L, et al. Interleukin-6, but not C-reactive protein, predicts the occurrence of cardiovascular events after drug-eluting stent for unstable angina. J Intervent Cardiol. 2014 Apr;27(2):142–54.

356. Akhabir L, Sandford A. Genetics of Interleukin 1 Receptor-Like 1 in Immune and Inflammatory Diseases. Curr Genomics. 2010 Dec;11(8):591–606.

357. Shah RV, Januzzi JL. ST2: a novel remodeling biomarker in acute and chronic heart failure. Curr Heart Fail Rep. 2010 Mar;7(1):9–14.

358. Kohli P, Bonaca MP, Kakkar R, Kudinova AY, Scirica BM, Sabatine MS, et al. Role of ST2 in non-ST-elevation acute coronary syndrome in the MERLIN-TIMI 36 trial. Clin Chem. 2012 Jan;58(1):257–66.

359. Luzina IG, Keegan AD, Heller NM, Rook GAW, Shea-Donohue T, Atamas SP. Regulation of inflammation by interleukin-4: a review of “alternatives.” J Leukoc Biol. 2012 Oct;92(4):753–64.

360. Sánchez Muñoz-Torrero JF, Rivas MD, Zamorano J, Alonso R, Joya-Vazquez P, Padró T, et al. rs1801275 Interleukin-4 receptor alpha polymorphism in familial hypercholesterolemia. J Clin Lipidol. 2014 Aug;8(4):418–22.

361. Liu Z, Zhang M, Wu J, Zhou P, Liu Y, Wu Y, et al. Serum CD121a (Interleukin 1 Receptor, Type I): A Potential Novel Inflammatory Marker for Coronary Heart Disease. PLOS ONE. 2015 Jun 22;10(6):e0131086.

362. Nishida H, Horio T, Suzuki Y, Iwashima Y, Kamide K, Kangawa K, et al. Plasma adrenomedullin as an independent predictor of future cardiovascular events in high-risk patients: Comparison with C-reactive protein and adiponectin. Peptides. 2008 Apr 1;29(4):599–605.

363. Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev. 2004 Jul;84(3):903–34.

364. Horio T, Nishikimi T, Yoshihara F, Matsuo H, Takishita S, Kangawa K. Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts. Eur J Pharmacol. 1999 Oct 1;382(1):1–9.

365. Calabro NE, Kristofik NJ, Kyriakides TR. Thrombospondin-2 and extracellular matrix assembly. Biochim Biophys Acta. 2014 Aug;1840(8):2396–402.

366. Berezin AE, Kremzer AA, Samura TA. Circulating thrombospondin-2 in patients with moderate-to-severe chronic heart failure due to coronary artery disease. J Biomed Res. 2016 Jan;30(1):32–9.

367. Youn B-S, Zhang S, Broxmeyer HE, Antol K, Fraser MJ, Hangoc G, et al. Isolation and Characterization of LMC, a Novel Lymphocyte and Monocyte Chemoattractant Human CC Chemokine, with Myelosuppressive Activity. Biochem Biophys Res Commun. 1998 Jun 18;247(2):217–22.

368. Prentice RL, Paczesny S, Aragaki A, Amon LM, Chen L, Pitteri SJ, et al. Novel proteins associated with risk for coronary heart disease or stroke among postmenopausal women identified by in-depth plasma proteome profiling. Genome Med. 2010 Jul 28;2(7):48.

369. Fülöp P, Seres I, Lőrincz H, Harangi M, Somodi S, Paragh G. Association of chemerin with oxidative stress, inflammation and classical adipokines in non-diabetic obese patients. J Cell Mol Med. 2014 Jul;18(7):1313–20.

370. İnci S, Aksan G, Doğan P. Chemerin as an independent predictor of cardiovascular event risk. Ther Adv Endocrinol Metab. 2016 Apr;7(2):57–68.

371. Ji Q, Lin Y, Liang Z, Yu K, Liu Y, Fang Z, et al. Chemerin is a novel biomarker of acute coronary syndrome but not of stable angina pectoris. Cardiovasc Diabetol. 2014 Nov 1;13:145.

372. Unsicker K, Spittau B, Krieglstein K. The multiple facets of the TGF-β family cytokine growth/differentiation factor-15/macrophage inhibitory cytokine-1. Cytokine Growth Factor Rev. 2013 Aug;24(4):373–84.

373. Lin J-F, Wu S, Hsu S-Y, Yeh K-H, Chou H-H, Cheng S-T, et al. Growth-differentiation factor-15 and major cardiac events. Am J Med Sci. 2014 Apr;347(4):305–11.

374. Wollert KC, Kempf T, Wallentin L. Growth Differentiation Factor 15 as a Biomarker in Cardiovascular Disease. Clin Chem. 2017 Jan;63(1):140–51.

375. Corre J, Hébraud B, Bourin P. Concise review: growth differentiation factor 15 in pathology: a clinical role? Stem Cells Transl Med. 2013 Dec;2(12):946–52.

376. Alam SR, Newby DE, Henriksen PA. Role of the endogenous elastase inhibitor, elafin, in cardiovascular injury: from epithelium to endothelium. Biochem Pharmacol. 2012 Mar 15;83(6):695–704.

377. Boraschi D, Tagliabue A. The interleukin-1 receptor family. Semin Immunol. 2013 Dec 15;25(6):394–407.

378. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ. Programmed Death Ligand 2 in Cancer-Induced Immune Suppression [Internet]. Journal of Immunology Research. 2012 [cited 2017 Oct 10]. Available from: https://www.hindawi.com/journals/jir/2012/656340/

379. Terai Y, Abe M, Miyamoto K, Koike M, Yamasaki M, Ueda M, et al. Vascular smooth muscle cell growth-promoting factor/F-spondin inhibits angiogenesis via the blockade of integrin alphavbeta3 on vascular endothelial cells. J Cell Physiol. 2001 Sep;188(3):394–402.

380. Zander CB, Cao W, Zheng XL. ADAMTS13 and von Willebrand factor interactions. Curr Opin Hematol. 2015 Sep;22(5):452–9.

381. Emmer BT, Ginsburg D, Desch KC. Von Willebrand Factor and ADAMTS13: Too Much or Too Little of a Good Thing? Arterioscler Thromb Vasc Biol. 2016 Dec 1;36(12):2281–2.

382. Wertel I, Surówka J, Polak G, Barczyński B, Bednarek W, Jakubowicz-Gil J, et al. Macrophage-derived chemokine CCL22 and regulatory T cells in ovarian cancer patients. Tumour Biol. 2015 Feb 3;36(6):4811–7.

383. Scott HS, Nelson PV, Litjens T, Hopwood JJ, Morris CP. Multiple polymorphisms within the alpha-L-iduronidase gene (IDUA): implications for a role in modification of MPS-I disease phenotype. Hum Mol Genet. 1993 Sep;2(9):1471–3.

384. Ikonomidis I, Michalakeas CA, Lekakis J, Paraskevaidis I, Kremastinos DT. Multimarker approach in cardiovascular risk prediction. Dis Markers. 2009;26(5–6):273–85.

385. Mayeux R. Biomarkers: Potential Uses and Limitations. NeuroRx. 2004 Apr;1(2):182–8.

386. Burness CE, Beacock D, Channer KS. Pitfalls and problems of relying on serum troponin. QJM Int J Med. 2005 May 1;98(5):365–71.

387. Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. Curr Opin Clin Nutr Metab Care. 2010 Sep;13(5):541–7.

388. Su C-W, Su B-F, Chiang W-L, Yang S-F, Chen M-K, Lin C-W. Plasma levels of the tissue inhibitor matrix metalloproteinase-3 as a potential biomarker in oral cancer progression. Int J Med Sci. 2017 Jan 1;14(1):37–44.

389. Kelly E, Owen CA, Abraham A, Knowlton DL, Celli BR, Pinto-Plata V. Comparison of arterial and venous blood biomarker levels in chronic obstructive pulmonary disease. F1000Research. 2013;2:114.

390. Zhang L, Peppel K, Sivashanmugam P, Orman ES, Brian L, Exum ST, et al. Expression of tumor necrosis factor receptor-1 in arterial wall cells promotes atherosclerosis. Arterioscler Thromb Vasc Biol. 2007 May;27(5):1087–94.

391. Maddahi A, Kruse LS, Chen Q-W, Edvinsson L. The role of tumor necrosis factor-α and TNF-α receptors in cerebral arteries following cerebral ischemia in rat. J Neuroinflammation. 2011 Aug 28;8:107.

392. Dey-Hazra E, Hertel B, Kirsch T, Woywodt A, Lovric S, Haller H, et al. Detection of circulating microparticles by flow cytometry: influence of centrifugation, filtration of buffer, and freezing. Vasc Health Risk Manag. 2010;6:1125–33.

393. Salgado Filho W, Filho M, E E, Horta P, Lemos PA, Migueletto BC, et al. Intracoronary inflammatory markers after percutaneous coronary interventions. Arq Bras Cardiol. 2005 Sep;85(3):180–5.

394. Lincoff AM, Kereiakes DJ, Mascelli MA, Deckelbaum LI, Barnathan ES, Patel KK, et al. Abciximab Suppresses the Rise in Levels of Circulating Inflammatory Markers After Percutaneous Coronary Revascularization. Circulation. 2001 Jul 10;104(2):163–7.

395. Preeshagul I, Gharbaran R, Jeong KH, Abdel-Razek A, Lee LY, Elman E, et al. Potential biomarkers for predicting outcomes in CABG cardiothoracic surgeries. J Cardiothorac Surg. 2013 Jul 18;8:176.

396. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell. 1999 Oct;4(4):563–71.

397. McConnell KJ, Olson KL, Delate T, Merenich JA, Clinical Pharmacy Cardiac Risk Service Study Group. Factors associated with recurrent coronary events among patients with cardiovascular disease. Pharmacotherapy. 2009 Aug;29(8):906–13.

398. Schulz R, Heusch G. Tumor necrosis factor-alpha and its receptors 1 and 2: Yin and Yang in myocardial infarction? Circulation. 2009 Mar 17;119(10):1355–7.

399. Grassi-Oliveira R, Brietzke E, Pezzi JC, Lopes RP, Teixeira AL, Bauer ME. Increased soluble tumor necrosis factor-α receptors in patients with major depressive disorder. Psychiatry Clin Neurosci. 2009 Apr 1;63(2):202–8.

400. Müller A, Krämer SD, Meletta R, Beck K, Selivanova SV, Rancic Z, et al. Gene expression levels of matrix metalloproteinases in human atherosclerotic plaques and evaluation of radiolabeled inhibitors as imaging agents for plaque vulnerability. Nucl Med Biol. 2014 Aug;41(7):562–9.

401. BOC Gene - GeneCards | BOC Protein | BOC Antibody [Internet]. [cited 2018 May 11]. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=BOC

402. Young SG, Fong LG. Lowering Plasma Cholesterol by Raising LDL Receptors—Revisited. N Engl J Med. 2012 Mar 22;366(12):1154–5.

403. Vuik SI, Mayer E, Darzi A. Enhancing risk stratification for use in integrated care: a cluster analysis of high-risk patients in a retrospective cohort study. BMJ Open [Internet]. 2016 Dec 19 [cited 2017 Sep 18];6(12). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5168666/

404. Sebastiani P, Thyagarajan B, Sun F, Schupf N, Newman AB, Montano M, et al. Biomarker signatures of aging. Aging Cell. 2017 Apr 1;16(2):329–38.

405. Ahlqvist E, Storm P, Käräjämäki A, Martinell M, Dorkhan M, Carlsson A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. Lancet Diabetes Endocrinol [Internet]. 2018 Mar 1 [cited 2018 Mar 5];0(0). Available from: http://www.thelancet.com/journals/landia/article/PIIS2213-8587(18)30051-2/abstract

406. Woodcock EA. Phospholipase C Signaling in Heart Disease. In: Phospholipases in Health and Disease [Internet]. Springer, New York, NY; 2014 [cited 2017 Sep 15]. p. 283–97. (Advances in Biochemistry in Health and Disease). Available from: https://link.springer.com/chapter/10.1007/978-1-4939-0464-8_17

407. Visweswaran S, Ferreira A, Ribeiro GA, Oliveira AC, Cooper GF. Personalized Modeling for Prediction with Decision-Path Models. PLOS ONE. 2015 Jun 22;10(6):e0131022.

408. Liu B, Taioli E. Seasonal Variations of Complete Blood Count and Inflammatory Biomarkers in the US Population - Analysis of NHANES Data. PLOS ONE. 2015 Nov 6;10(11):e0142382.

409. Marti-Soler H, Gubelmann C, Aeschbacher S, Alves L, Bobak M, Bongard V, et al. Seasonality of cardiovascular risk factors: an analysis including over 230 000 participants in 15 countries. Heart Br Card Soc. 2014 Oct;100(19):1517–23.

410. Rudnicka AR, Rumley A, Lowe GDO, Strachan DP. Diurnal, seasonal, and blood-processing patterns in levels of circulating fibrinogen, fibrin D-dimer, C-reactive protein, tissue plasminogen activator, and von Willebrand factor in a 45-year-old population. Circulation. 2007 Feb 27;115(8):996–1003.

411. Rosenthal NE, Sack DA, Gillin JC, Lewy AJ, Goodwin FK, Davenport Y, et al. Seasonal affective disorder. A description of the syndrome and preliminary findings with light therapy. Arch Gen Psychiatry. 1984 Jan;41(1):72–80.

412. Gallerani M, Govoni M, Mucinelli M, Bigoni M, Trotta F, Manfredini R. Seasonal variation in the onset of acute microcrystalline arthritis. Rheumatol Oxf Engl. 1999 Oct;38(10):1003–6.

413. Lewington S, Li L, Sherliker P, Guo Y, Millwood I, Bian Z, et al. Seasonal variation in blood pressure and its relationship with outdoor temperature in 10 diverse regions of China: the China Kadoorie Biobank. J Hypertens. 2012 Jul;30(7):1383–91.

414. Fares A. Winter Cardiovascular Diseases Phenomenon. North Am J Med Sci. 2013 Apr;5(4):266–79.

415. Pell JP, Cobbe SM. Seasonal variations in coronary heart disease. QJM Mon J Assoc Physicians. 1999 Dec;92(12):689–96.

416. Nakao M, Yamauchi K, Ishihara Y, Omori H, Ichinnorov D, Solongo B. Effects of air pollution and seasons on health-related quality of life of Mongolian adults living in Ulaanbaatar: cross-sectional studies. BMC Public Health. 2017 Jun 23;17:594.

417. Peters A, Dockery DW, Muller JE, Mittleman MA. Increased Particulate Air Pollution and the Triggering of Myocardial Infarction. Circulation. 2001 Jun 12;103(23):2810–5.

418. Kinlay S, Libby P, Ganz P. Endothelial function and coronary artery disease. Curr Opin Lipidol. 2001 Aug;12(4):383–9.

419. Anderson TJ, Gerhard MD, Meredith IT, Charbonneau F, Delagrange D, Creager MA, et al. Systemic nature of endothelial dysfunction in atherosclerosis. Am J Cardiol. 1995 Feb 23;75(6):71B–74B.

420. Hadi HA, Carr CS, Al Suwaidi J. Endothelial Dysfunction: Cardiovascular Risk Factors, Therapy, and Outcome. Vasc Health Risk Manag. 2005 Sep;1(3):183–98.

421. Tasev D, Koolwijk P, van Hinsbergh VWM. Therapeutic Potential of Human-Derived Endothelial Colony-Forming Cells in Animal Models. Tissue Eng Part B Rev. 2016 Oct;22(5):371–82.

422. Medina RJ, Barber CL, Sabatier F, Dignat-George F, Melero-Martin JM, Khosrotehrani K, et al. Endothelial Progenitors: A Consensus Statement on Nomenclature. Stem Cells Transl Med. 2017 May;6(5):1316–20.

423. De Dios I, Ramudo L, Alonso JR, Recio JS, Garcia-Montero AC, Manso MA. CD45 expression on rat acinar cells: Involvement in pro-inflammatory cytokine production. FEBS Lett. 2005 Nov 21;579(28):6355–60.